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1982

ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF GENERAL MEDICAL SCIENCES
Fiscal Year 1982

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THE DIRECTOR'S REPORT

As Fiscal Year 1982 comes to a close, the National Institute of General Medical Sciences (NIGMS) prepares to mark, in October, the twentieth anniversary of the passing of legislation which established it as one of the National Institutes of Health. It seems appropriate, therefore, to reaffirm the commitment upon which the Institute was established, namely, the support of the highest quality research and research training in the basic medical sciences. Through such support, NIGMS seeks to expand knowledge about the fundamental life processes that underlie human health and disease.

During the past year, the ability to award research and research training grants to all those whose studies have been judged to have the highest scientific merit has been greatly restricted, and the level of funds provided has been reduced below that recommended as needed to perform these studies, even though larger sums of money had been previously committed to these projects. The Institute staff has exercised careful thought and used superb judgment in assessing each individual award and preparing the specific reductions with great care so as to perturb grantees to the least extent possible. Nevertheless, it is painfully apparent that, at the very moment in time when the most exciting discoveries are being made in the fields of genetics, cellular biology, molecular biology and molecular pharmacology, progress may be slowed by the reduction in funds. This is particularly serious because the studies supported by NIGMS are absolutely vital to, and at the forefront of, modern-day biomedical science. Only from such fundamental studies will leads come which are related to the diagnosis, prevention, treatment and cure of the multitude of diseases not yet understood, specific studies of which are within the missions of the categorical institutes.

Fiscal year 1982 saw many changes at NIH as a whole, which had an impact on NIGMS. During the nine months that Dr. Thomas Malone was Acting Director of NIH, he strongly endorsed the NIGMS emphasis on support of investigator-initiated research and predoctoral research training. In reviewing the Institute's programs for the new Director, NIH, Dr. James Wyngaarden, we reassessed our major activities and concluded that, in addition to individual research grants, and predoctoral research training grants, increased support for individual postdoctoral fellowships should be emphasized, if funds permit.

Furthermore, the Institute is and must continue to be responsive to those specific needs of its grantees which will enable more rapid progress to be made. Thus, after a year or more of deliberations and consultation with molecular biologists both in the United States and Europe, NIGMS has awarded a research contract to establish a Nucleic Acid Sequence Data Bank, from which all investigators may rapidly obtain information regarding known nucleotide sequences, and into which new sequence data may be added as it is developed. This contract is being funded by NIGMS in cooperation with a number of other NIH institutes (National Institute of Allergy and Infectious Diseases, National Cancer Institute, Division of Research Resources) and with other government agencies (National Science Foundation, Department of Energy, Department of Defense). Such a collaborative venture is unique.

During this year, significant changes in the Institute's personnel occurred. After serving as the Associate Director in charge of grant activities for nine years, and with the Institute for thirteen years, Dr. Arthur Heming retired in

December 1981. Dr. Elke Jordan, previously Deputy Director of the Genetics Program, was appointed as Associate Director for Program Activities early in 1982 and has already gained the respect and admiration of all for her skills in handling the ever-changing directives under which NIGMS grants have to be made. The Institute's projected plans to better retrieve data regarding grant awards and expenditures through the use of computerization are underway under the leadership of Mr. Thomas Mitchell who returned to NIGMS where his career started ten years ago.

Office of Review Activities

In addition to the four established review committees (Cellular and Molecular Basis of Disease, Genetic Basis of Disease, Pharmacological Sciences, and Minority Access to Research Careers Review Committees), several *ad hoc* groups have also been used.

The combined review load of the office is shown below:

Category	Number of Applications	Requested Dollars (in millions)
Training Grants	97	$99.1
Research Centers	7	18.9
Program Projects	6	10.9
MARC Fellowships	51	(dollars not available)

Staff Changes: The following individuals were added to the staff:

Dr. James Cassatt - Genetics Program
Dr. James Gilliam - Pharmacological Sciences Program
Dr. Judith Greenberg - Genetics Program
Mr. Thomas Mitchell - Office of Program Analysis
Dr. Helen Sunshine - Office of Review Activities

The following staff members left the Institute:

Dr. Matti Al-Aish
Dr. Roger Fuson
Dr. Arthur Heming
Dr. Robert Wenthold

National Advisory General Medical Sciences Council

Three new Council members were appointed for four year terms: Drs. Harry Oberhelman of Stanford University, Daniel Tosteson of Harvard University, and Peter von Hippel of the University of Oregon. The terms of Dr. Francisco Ayala and Ms. Joanne Mizner expired at the end of FY 1982.

FY 1982 TOTAL SUPPORT BY ACTIVITY/GRANT TYPE/GRANT NUMBER AND SHOWING PROGRAM AREAS

(Dollars in Thousands)

		CMBD		GEN		PS		PBME		MARC		TOTAL	
		NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS
RESEARCH CENTERS													
P50	TYPES 5 & 7	1	596	4	2,880	5	3,446	10	3,643	--	--	20	10,567
	TYPES 2 & 9	--	--	1	525	--	--	1	549	--	--	2	1,074
	TYPE 3*	--	--	--	--	(1)	102	(2)	28	--	--	(3)	131
Subtotal		1	596	5	3,305	5	3,549	11	4,222	--	--	22	11,773
RESEARCH PROJECTS													
P01	TYPES 5 & 7	7	3,267	9	4,687	2	613	5	2,897	--	--	23	11,465
	TYPES 2 & 9	--	--	--	--	4	1,320	2	723	--	--	6	2,044
	TYPE 1	1	426	--	--	--	--	2	1,196	--	--	3	1,623
	TYPE 3*	(3)	415	(1)	269	(1)	9	--	--	--	--	(5)	694
	Subtotal	8	4,108	9	4,957	6	1,944	9	4,817	--	--	32	15,827
P41	TYPES 5 & 7	--	--	--	--	--	--	3	48	--	--	3	48
R01	TYPES 5 & 7	642	58,045	715	72,459	205	17,311	140	12,815	--	--	1,702	160,632
	TYPES 2 & 9	159	17,193	174	20,576	68	7,433	40	5,218	--	--	441	50,422
	TYPE 1	137	11,196	142	13,280	51	4,410	39	4,253	--	--	369	33,141
	TYPE 3*	(13)	597	(20)	1,101	(7)	429	(3)	158	--	--	(43)	2,287
	Subtotal	938	87,032	1,031	107,418	324	29,585	219	22,446	--	--	2,512	246,483
R23	TYPES 5 & 7	--	--	--	--	--	--	11	480	--	--	11	480
	TYPE 1	--	--	--	--	--	--	4	181	--	--	4	181
	Subtotal	--	--	--	--	--	--	15	662	--	--	15	662
Subtotal		946	91,140	1,040	112,376	330	31,529	246	27,973	--	--	2,562	263,020
OTHER RESEARCH													
R13	TYPES 5 & 7	1	95	1	30	--	--	--	--	--	--	2	126
	TYPE 1	5	12	7	22	1	2	--	--	--	--	13	36
Subtotal		6	107	8	52	1	2	--	--	--	--	15	162
TOTAL RESEARCH GRANTS		953	91,846	1,053	115,834	336	35,080	257	32,195	1	73	2,599	274,956

* Counts of supplements to existing awards are enclosed in ( ).

NOTE: Discrepancies in totals due to rounding.

FY 1982 TOTAL SUPPORT BY ACTIVITY/GRANT TYPE/GRANT NUMBER AND SHOWING PROGRAM AREAS
(Dollars in Thousands)

	CMBD		GEN		PS		PBME		MARC		TOTAL	
	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS
CAREER AWARDS												
K04 TYPES 5 & 7	1	41	1	40	2	77	6	234	--	--	10	394
K06 TYPES 5 & 7	7	229	7	233	1	35	4	130	--	--	19	628
Total Career Awds	8	270	8	274	3	112	10	364	--	--	29	1,022
INDIVIDUAL FELLOWSHIPS												
F31 TYPES 5 & 7	--	--	--	--	--	--	--	--	1	9	1	9
TYPE 1	--	--	--	--	--	--	--	--	10	90	10	90
Subtotal	--	--	--	--	--	--	--	--	11	99	11	99
F32 TYPES 5 & 7	54	892	94	1,595	19	318	6	110	--	--	173	2,919
TYPES 2 & 9	1	17	2	32	--	--	--	--	--	--	3	49
TYPE 1	61	1,018	79	1,312	17	284	5	87	--	--	162	2,702
TYPE 3*	(3)	3	(17)	16	(1)	0.5	--	--	--	--	(21)	20
Subtotal	116	1,932	175	2,956	36	603	11	197			338	5,690
F33 TYPES 5 & 7	--	--	1	8	--	--	--	--	--	--	1	8
TYPE 1	--	--	4	121	--	--	--	--	--	--	4	121
Subtotal	--	--	5	129	--	--	--	--	--	--	5	129
F34 TYPES 5 & 7	--	--	--	--	--	--	--	--	10	210	10	210
TYPE 1	--	--	--	--	--	--	--	--	11	258	11	258
Subtotal	--	--	--	--	--	--	--	--	21	468	21	468
Total Individual Fellowships	116	1,932	180	3,085	36	603	11	197	32	567	375	6,387

* Counts of supplements to existing awards are enclosed in ( ).

NOTE: Discrepancies in totals due to rounding.

FY 1982 TOTAL SUPPORT BY ACTIVITY/GRANT TYPE/GRANT NUMBER
AND SHOWING PROGRAM AREAS
(Dollars in Thousands)

	CMBD		GEN		PS		PBME		MARC		TOTAL	
	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS
INSTITUTIONAL TRAINING AWARDS												
T32 TYPES 5 & 7	64	18,597	44	5,276	37	3,289	32	2,513	4	531	181	30,208
TYPES 2 & 9	11	1,738	14	1,414	9	655	9	983	--	--	43	4,792
TYPE 1	2	64	--	--	2	169	1	23	--	--	5	256
TYPE 3*	--	--	--	--	--	--	(1)	29	--	--	(1)	29
Subtotal	77	20,401	58	6,691	48	4,114	42	3,549	4	531	229	35,286
T34 TYPES 5 & 7	--	--	--	--	--	--	--	--	27	2,701	27	2,701
TYPES 2 & 9	--	--	--	--	--	--	--	--	9	1,155	9	1,155
TYPE 1	--	--	--	--	--	--	--	--	4	339	4	339
Subtotal	--	--	--	--	--	--	--	--	40	4,196	40	4,196
T35 TYPES 5 & 7	--	--	--	--	--	--	5	75	--	--	5	75
T36 TYPE 1	--	--	--	--	--	--	--	--	1	73	1	73
Total Institutional Training	77	20,401	58	6,691	48	4,114	47	3,624	45	4,801	275	39,632
TOTAL GRANTS (Research, Career Awards, Individual Fellowships, Institutional Training Awards)	1,154	114,451	1,299	125,885	423	39,910	325	36,382	77	5,369	3,278	321,999

* Counts of supplements to existing awards are enclosed in ( ).

NOTE: Discrepancies in totals due to rounding.

FY 1982 NATIONAL RESEARCH SERVICE AWARDS TOTAL SUPPORT BY ACADEMIC LEVEL/ACTIVITY/ANNOUNCED AREA

(Dollars in Thousands)

ANNOUNCED	TYPES 5 & 7		TYPES 2 & 9		TYPE 1		TYPE 3*		TOTAL	
AREA	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS
Predoctoral Individual (F31)										
MARC	1	9	--	--	10	90	--	--	11	99
Predoctoral Individual (F34)										
MARC	8	154	--	--	7	165	--	--	15	319
MARC [Paid by Other Insts.]	[2]	[46]	[1]	[18]	[2]	[44]	--	--	[5]	[109]
Subtotal	9	163	--	--	17	255			26	418
Postdoctoral Individual (F33)										
GENETICS	1	8	--	--	4	120	--	--	5	128
Total, F33's	1	8	--	--	4	120	--	--	5	128
Postdoctoral Individual (F32, F34)										
CELL-MOLE. BIO.	54	892	1	17	61	1,018	(3)	3	116(3)	1,932
GENETICS	94	1,595	2	32	79	1,312	(17)	16	175	2,956
CLIN PHARM.	--	--	--	--	1	17	--	--	1	17
PHARM-SCI.	19	318	--	--	16	266	(1)	0.5	35(1)	585
SYS & INT BIO.	5	91	--	--	4	68	--	--	9	160
TRAUMA	1	19	--	--	--	--	--	--	1	19
ANESTH.	--	--	--	--	--	--	--	--	--	--
BEHAVIORAL SCI.	--	--	--	--	1	18	--	--	1	18
MARC	2	56	--	--	4	93	--	--	6	149
MARC [Paid by Other Insts.]	[3]	[67]	--	--	[2]	[46]	[2]	[678]	[5] [2]	[114]
Total, F32's, F34's	175	2,973	3	49	166	2,795	(21)	20	344	5,839

* Counts of supplements to existing awards are enclosed in ( ).

NOTE: Discrepancies in totals due to rounding.

FY 1982 NATIONAL RESEARCH SERVICE AWARDS TOTAL SUPPORT
BY ACADEMIC LEVEL/ACTIVITY/ANNOUNCED AREA
(Dollars in Thousands)

ANNOUNCED AREA	TYPES 5 & 7			TYPES 2 & 9			TYPE 1			TYPE 3*			TOTAL		
	NO.	POS.	DOLLARS	NO.	POS.	DOLLARS	NO.	POS.	DOLLARS	NO.	POS.	DOLLARS	NO.	POS.	DOLLARS
							Predoctoral Institutional (T35)								
SHORT-TERM RESEARCH TRAINING	5	54	75	--	--	--	--	--	--	--	--	--	5	54	75
							Predoctoral Institutional (T32)								
CELL-MOLE. BIO.	40	870	9,952	4	85	831	--	--	--	--	--	--	44	955	10,784
GENETICS	30	413	4,364	7	74	844	--	--	--	--	--	--	37	487	5,209
PHARM-SCI.	33	300	3,067	6	47	432	1	16	150	--	--	--	40	363	3,650
SYS & INT. BIO.	20	177	1,786	7	81	900	1	2	23	(1)	--	29	28	260	2,739
MED-SCI.	18	610	8,201	4	63	777	1	3	20	--	--	--	23	676	8,999
MARC	31	268	3,232	9	78	1,155	5	18	413	--	--	--	45	364	4,801
Total, Pre-doc. T32's	177	2692	30,680	37	428	4,941	8	39	607	(1)	--	29	222	3159	36,259
							Postdoctoral Institutional (T32)								
PATHOBIO.	6	24	443	3	9	129	1	2	43	--	--	--	10	35	617
GENETICS	14	53	912	7	30	569	--	--	--	--	--	--	21	83	1,481
CLIN PHARM	4	11	221	3	13	222	1	1	19	--	--	--	8	25	463
TRAUMA	7	25	494	2	4	83	--	--	--	--	--	--	9	29	577
ANESTH.	5	13	232	--	--	--	--	--	--	--	--	--	5	13	232
Total, Post-doc. T32's	36	126	2,304	15	56	1,005	2	3	62	--	--	--	53	185	3,373

* Counts of supplements to existing awards are enclosed in ( ).

NOTE: Discrepancies in totals due to rounding.

FY 1982 PBME GRANT SUPPORT IN SELECTED AREAS
BY PROGRAM/ACTIVITY/GRANT TYPE
(Dollars in Thousands)

PROGRAM AREA	ACTIVITY	TYPES 5 & 7		TYPES 2 & 9		TYPE 1		TYPE 3*		TOTAL	
		NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS
ANESTHESIOLOGY	K04	3	115	--	--	--	--	--	--	3	115
	P50	1	338	--	--	--	--	--	--	1	338
	R01	21	1,552	5	374	4	397	--	--	30	2,324
	R23	6	240	--	--	2	86	--	--	8	327
Total Anesth.		31	2,247	5	374	6	484	--	6	42	3,105
BIOENGINEERING	K04	1	41	--	--	--	--	--	--	1	41
	K06	3	96	--	--	--	--	--	--	3	96
	P01	5	2,897	1	346	1	746	--	--	7	3,989
	P41	3	48	--	--	--	--	--	--	3	48
	R01	86	8,044	25	3,493	25	2,912	(3)	158	136	14,610
Total Bioeng.		98	11,128	26	3,840	26	3,658	(3)	158	150	18,786
TRAUMA	K04	2	76	--	--	--	--	--	--	2	76
	K06	1	33	--	--	--	--	--	--	1	33
	P01	--	--	1	376	1	450	--	--	2	827
	P50	9	3,305	1	549	--	--	(2)	28	10	3,883
	R01	33	3,217	10	1,350	10	943	--	--	53	5,511
	R23	5	240	--	--	2	94	--	--	7	334
Total Trauma		50	6,873	12	2,277	13	1,487	(2)	28	75	10,667
Total (PBME)		179	20,250	43	6,491	45	5,630	(5)	187	267	32,559

*Counts of supplements to existing awards are enclosed in ( ).
NOTE: Discrepancies in totals due to rounding.

OFFICE OF THE ASSISTANT DIRECTOR FOR CLINICAL RESEARCH

Fiscal year 1982 was a year for the Assistant Director for Clinical Research to plan and to participate in conferences related to trauma and burn research, and also in her dual role as program administrator of the Physiology and Biomedical Engineering (PBME) Program to observe a number of reviews of large grants.

1. Research Center and Program Project Proposals

As a result of the announcement by the Institute in the NIH Guide for Grants and Contracts, Vol. 10, No. 4, March 6, 1981, numerous inquiries and visits to the Institute were made by potential applicants. Since it is recognized that the preparation of an application for a large multi-investigator grant requires a substantial investment of time, effort, and resources, consultation with the NIGMS staff before formal submission of such an application allows staff to determine whether or not the proposed research fits the mission of the Institute. As a result of these discussions, eight large grant proposals were actually submitted.

During FY 1982, three large research grant applications were considered to be of high scientific merit based on peer review and were funded by the Institute. One of these is a program project headed by Dr. Gary Striker at the University of Washington. This very basic program is an outgrowth of the more applied research supported through the research center grant under the leadership of Dr. James Carrico at the University of Washington. The focus of the program project is in the area of cellular and molecular responses to injury of small blood vessels. Also emphasis is placed on the cell population in pulmonary alveoli and its relationship to pulmonary failure, a major cause of death following severe trauma.

Currently the Institute supports nine research centers and three program projects in trauma and burns. Each program is unique in some aspects of its work. Emphasis is placed on such research areas as metabolism, infection, organ failure, and wound healing.

The Assistant Director and PBME Program staff visited the trauma and burn units of the Washington Hospital Center. These are very active emergency programs in the Washington, D.C. area. Dr. Howard Champion, director of the trauma unit, described his system for measuring severity and extent of injury. The thrust of the program is toward delivery of health care, improvement of its methodology, and application of pre-established indices. Dr. Marion Jordan, director of the burn unit, attended the NIGMS 1978 Consensus Conference as well as the 1980 follow-up Conference on Supportive Therapy in Burn Care, and is currently applying a number of the consensus items to the care of burned patients.

2. Presentations and Meetings

The American Burn Association annual meeting was held in Boston, Massachusetts, May 12-15, 1982. Dr. John F. Burke, an NIGMS research center grantee (P50 GM 21700), gave the presidential address to the Association. Dr. Burke presented the progress on his work with Dr. Ioannis Yannas, another NIGMS grantee (R01 GM 23946), on artificial skin (first reported on in the 1980 Annual Report). They are concentrating their research on Stage II in the development of the artificial skin in an effort to reduce the time of the skin enclosure, to avoid the use of autografts for epidermal growth, and to reduce contracture and deep scarring. The team of investigators are seeding the two layer polymeric membrane with a small quantity of uncultured autogenous epidermal (basal) cells prior to grafting.

Following grafting of the wound with the seeded membrane, the basal cells proliferate at the interface of the two layers forming sheets of mature neoepidermis. The Stage II membrane is not limited in size and does not need a second procedure to be effective. So far Stage II membrane has only been used on animals. The team found that it is possible to graft animals with seeded membranes within four hours of harvesting the cells. Animal studies continue to be effective with minimal scarring since new dermis and epidermis are being made instead of scar tissue.

The Institute was well represented at the American Burn Association meeting. Approximately one-fourth of the papers were presented by NIGMS grantees. The Assistant Director for Clinical Research was awarded the Harvey Stuart Allen Distinguished Service Award for "dedication to quality and her imaginative assistance to developing research in burn injury and improvement in burn treatment, which has won the admiration of all."

The Institute has continued to receive world wide requests for copies of the proceedings of the 1978 Consensus Development Conference on Supportive Therapy in Burn Care and the 1980 follow-up conference. A third conference is planned for September 1983, on new frontiers in the understanding of burn injuries. This conference will also be sponsored by the International Society for Burn Injuries and the World Health Organization.

Every four years, the International Society for Burn Injuries holds a scientific congress. The Sixth Congress will be held in San Francisco, California, August 29 to September 4, 1982. The Assistant Director, together with Drs. John Burke and Charles Baxter, will present a paper summarizing the 1978 consensus conference and the 1980 follow-up conference on supportive therapy in burn care. Hopefully, this will set the stage for the 1983 conference. The Institute plans to have a scientific exhibit at the Congress highlighting research supported by the Institute.

CELLULAR AND MOLECULAR BASIS OF DISEASE PROGRAM

PROGRAM SCOPE

The cell is the fundamental biological unit of all living organisms. It holds the capacity for generating, from simple exogenous sources, all its macromolecules, energy, and complex organization and structure. Cells form selective cell-cell associations, continually displaying highly specific intercommunication and interacting with and adapting to environmental factors. Although they start from a single zygote, cells differentiate into a remarkable range of specialized types. The selective expression of the genome in differentiated cells and numerous factors, both internal and external (including interactions with other cells), bring this about.

This differentiation produces an enormous variety of cells in the tissues of higher organisms. Many cells have been cultured, often through many generations. These defined cell lines have proven of immense research value and supplement studies on primary cell cultures as well as cells *in vivo* or in intact tissues. Additionally, microorganisms, both prokaryotic and eukaryotic, have distinct advantages, especially in ease of culture and manipulation. Considering the overwhelming variety of life, it is not surprising that normal human tissues are often not used in basic investigations. Moreover, certain experimental preparations have been studied so extensively over extended periods that they are used because of the wealth of accumulated knowledge of their behavior. However, the research frequently progresses to human tissues to answer questions regarding normal human function and disease.

A major discovery of the past half-century has been the existence of subcellular organelles and the revelation of their variety of structure and function. The advent of sophisticated microscopic and other optical techniques has allowed us to visualize these organelles in fine, even molecular, detail.

Organelles have been isolated and, in some cases, disassembled and reconstructed *in vitro*. Much of the focus of the program has been on the role of individual organelles. The Cellular and Molecular Basis of Disease Program of the National Institute of General Medical Sciences supports research into mitochondria, endoplasmic reticulum, the Golgi apparatus, microtubules, microfilaments, and the plasma membrane.

An example of the scope of the program's support, is the support of research on plasma membranes. In this program are significant numbers of research grants on: plasma membrane biogenesis and destruction; interactions between membranes of different cells; cell anatomy; membrane physiology, including transmembrane transport; membrane-localized enzymes; the physical chemistry of lipids; primary and tertiary structures of membrane proteins; membrane protein molecular biology; the physics of lipids and lipid bilayers, including the forces holding bilayers together; bilayer electrical properties; and the physical state of lipids in living tissues.

As can be glimpsed from this brief outline, this program supports a wide range of investigations into cells and their components. It draws on techniques from chemistry, physics, and biochemistry. Its grantees may be in departments of physics, chemistry, immunology, microbiology, physiology, or anatomy, among others. As principally a supporter of investigator initiated research, the CMBD Program is limited only by the vision of high quality investigators of the cell and its component organelles and molecules.

OBJECTIVE

The Program's objective is to gain the fullest knowledge about human cells to assist in the prevention, treatment, and cure of disease in man. Since living cells generally share fundamental biological, chemical, and physical processes and properties, the Cellular and Molecular Basis of Disease Program supports research using cell and molecular systems from a wide variety of species, tissues, and preparations. The basis of selection is the scientific merit of research using preparations best suited to solving general principles and problems. In pursuit of this objective, the program supports research ranging from precise physical and mathematical approaches in the study of enzyme catalyzed reactions to investigation of the cellular interactions during embryonic development. Results from this broad range of research are reported in many scientific journals, adding important general principles, techniques, and information which contribute directly and indirectly to our expanded knowledge about normal and abnormal cellular functions. It is this fundamental knowledge that undergirds the study of human function and disease.

PROGRAM ORGANIZATION

The overall CMBD research program is administered by a Director and Deputy Director, and nine other professional staff members. It is organized into two sections and subprogram areas. The professional staff is:

Charles A. Miller, Ph.D.	Director
Vincent E. Price, M.D.	Deputy Director
Vivian F. Dickson	Special Assistant

Cellular Basis of Disease Section:

Bert I. Shapiro, Ph.D.	Chief
Artrice V. Bader, Ph.D.	Program Administator
W. Sue Badman, Ph.D.	Program Administator

Molecular Basis of Disease Section:

Marvin Cassman, Ph.D.	Chief
David P. Beck, Ph.D.	Program Administrator
Warren C. Jones, Ph.D.	Program Administrator
Lee Van Lenten, M.D.	Program Administator
John C. Norvell, Ph.D.	Program Administrator

Research training is administered outside the section organization. Drs. Norvell, Price, and Bader are responsible for training.

CELLULAR BASIS OF DISEASE SECTION

The emphasis of the Cellular Basis of Disease Section is research on the cell and its subcellular components, including basic research on all types of cells (i.e., prokaryotic and eukaryotic microorganisms, cells in tissue or organ culture, isolated cells, such as blood cells, sperm, and fertilized and unfertilized ova), as well as research focused on specific cells and their function in excised and intact tissues and organisms. Questions and problems explored and pertaining to specific cell types or components are of a fundamental or general nature applicable to other cell types. This section includes the support of research on all classes of basic cellular or subcellular research not expressly directed to the disease-oriented mission of a single categorical Institute, as well as cell biology related to several disease or general pathological states.

A large fraction of the research grants in this program, and an increasing number of new proposals, falls into two major categories: studies on cell membranes and studies on cellular motility and the cytoskeleton. The emphasis of the membrane program is on the plasma membrane of cells, but a significant fraction of the effort is devoted to intracellular membranes. The major areas include active and other transmembrane transport, membrane structure, membrane proteins, and membrane biogenesis. The research on cell motility involves studies of motion in cells, ranging from gross cellular movements such as protoplasmic streaming to the bending of cilia and flagella. Emphasis is on movement of and within non-muscle cells. The larger portion of the work supported here involves studies of cell structures such as microtubules and microfilaments; the proteins associated with motile structures, including microtubular-associated proteins, tubulin, myosin, paramyosin, actin and kinases; and, movement during cell division, including the nature of the mitotic spindle as well as the forces generated during division.

Control of other events during the cell cycle is also supported in this section. Questions addressed include the regulation and synchronization of protein and membrane synthesis, and the development of organelles. The program supports a significant number of projects concerned with the mechanism of cell differentiation. Consistent with the Institute's mission to support basic research, the work on development does not necessarily involve mammalian cells. It does include investigations into a multiplicity of systems, ranging from slime molds to tissue culture, sea urchin eggs to multicellular bacterial systems.

Many other areas are represented to some extent in the Cell Section of the program. Among these are cellular immunology and immunobiology, mechanisms of cell fusion, studies of cell adhesion, the role of membrane-bound enzymes, and lipid biochemistry. The latter includes measurements of lipid viscosity, enzymatic reactions in lipid in both its liquid and semisolid phases, and the conditions for micellar and vesicular formation.

MOLECULAR BASIS OF DISEASE SECTION

The Molecular Basis of Disease Section emphasizes support of basic research on: enzymology and regulatory processes in intermediary metabolism; structure and function of the enzyme active site, including the involvement of coenzymes and metal ions; mechanisms of oxidative phosphorylation and electron transport as well as the coupling of these processes to other cellular events such as transmembrane transport; physical and chemical studies on the ordered structure of proteins; and theoretical analyses and modeling of systems of biological importance. Essentially all areas of basic biochemistry and physical chemistry of general biomedical significance are included.

A large fraction of the research grants is concerned with enzyme and protein structure and the relationship to function. The program includes many projects on the nature of the active site. Another significant group includes the analysis of metabolic regulation by covalent modification; for example, cAMP-dependent phosphorylation reactions. Many grantees focus on determining primary, secondary, and tertiary protein structures. These projects employ the use of many state-of-the-art sophisticated techniques, including diffraction analysis and spectroscopic measurements. Among the proteins studied in detail are glycoconjugates and the proteins in the electron-transport chain. In this case the spatial organization of the complex of electron transport components involved in ATP synthesis is a subject of intensive study. The level of complexity being studied by such detailed molecular analysis makes the task formidable.

Although not a large fraction of the Molecular Basis of Disease Program, the theoretical research is a valued part of the section. Some studies of great interest address the question of the forces controlling the folding of proteins and the acquisition of stable folded conformations. Similar studies utilizing advanced methods of quantum chemistry are directed at other specific problems such as antibody-antigen interactions and enzymatic reactions. Research on the structure, molecular dynamics and physical properties of water, electrolytes, and hydrogen bonds is also supported.

Much of the research in the Molecular Section involves extensive use of sophisticated instrumentation. Often, the highly quantitative procedures used are at the frontiers of technical capabilities. Techniques utilized are nuclear and electron spin resonance, fluorescence, Mossbauer spectroscopy, circular dichroism and magnetic circular dichroism, Raman spectroscopy, X-ray, neutron and electron diffraction, and computer analysis, data reduction and modeling. The instrumental capabilities developed on research projects supported in this program often are extended to a wide variety of other areas of biomedicine. The most clinical areas of research and even medical practice may utilize technologies and instruments developed by basic scientists working on physical and chemical approaches to biomedical problems.

RESEARCH TRAINING

The Cellular and Molecular Basis of Disease Program (CMBD) supports predoctoral and postdoctoral research training in a broad range of disciplines. Approximately one-half of the total Institute training effort is administered by the CMBD Program. In turn, this program alone accounts for well over one-third of the predoctoral trainees supported by all of the NIH, inclusive of the Medical Scientist (MD/Ph.D. degree) training grant program. The very high percentage of doctoral level graduates from among these CMBD trainees (over 1600 this year at first-rate graduate and medical schools) constitute a major pool of the newly developing investigators for all Institutes of the National Institutes of Health.

Postdoctoral support, provided both through individual fellowships and institutional NRSA training grants, is essential for the long-term mission of the

NIGMS and numerous other NIH research efforts since it fosters the establishment of a cadre of first-class young scientists in all disciplines and areas comprising cellular and molecular biology. These scientists will be among the biomedical leaders in research supported throughout the NIH over the next several decades.

1. Cellular and Molecular Biology Institutional Predoctoral Training Program

The Cellular and Molecular Biology research training grants are unique in their broad coverage of the biomedical sciences, with faculty and students from many academic departments and/or disciplines, such as biochemistry, biophysics, microbiology, cell biology, molecular biology, genetics, anatomy, pathology, physics, chemistry, physiology, pharmacology, neurobiology and endocrinology. These grants provide support for the highest caliber trainees in these fields guided by productive faculty scientists in 44 separate university programs across the country. A total of 955 trainee positions are available in the 44 programs funded in Fiscal Year 1982. Twenty-five of these research training grants were awarded to private universities and 19 to public institutions in 23 states. The grants range in size from 4 predocotral trainee positions to 57 positions.

During this year, ten ongoing training programs were evaluated for renewal. Four of these competitive renewal applications were awarded; one was transferred to the NIGMS Genetics Institutional Predoctoral Training Program where it was awarded as a combined grant with an application from the same institution; and five were not funded. No new grants were made in the CMBD Program and it was not not possible to provide the full recommended number of trainee positions. Thus, the overall size was reduced by 8 grants and 83 trainees compared to Fiscal Year 1981. Even so, essentially all of the funded universities were able to select some new outstanding Ph.D. degree candidates for stipend and tuition support this year as earlier trainees moved to other available support or graduated.

Various patterns of training grant support, of eight months or more for each student, are utilized to gain the maximum benefit of limited funds toward providing for the recommended number of trainees. This has allowed, on the average, two years of support for each trainee during their four to six years as Ph.D. degree candidates. Such support, completely free of other obligations, is the fundamental purpose of the training grant--to provide each top student with a period to maximally develop independent and unique scholarship, and an original thesis research project. Essentially all recent graduates of this program are pursuing advanced research training in areas complementary to their Ph.D. degree work as postdoctoral fellows or research associates in excellent laboratories throughout the nation and abroad.

2. Medical Scientist Institutional Predoctoral Training Program

The goal of the Medical Scientist Program is to assist medical schools and their universities in the development and operation of special programs of combined scientific and medical training for highly motivated students of outstanding research and academic potential. Each institution is encouraged to utilize its unique strengths in developing its program, and each candidate's training over a six to seven year period draws, as needed, on the full range of scientific and medical opportunities available within the institution.

Trainees are awarded the combined MD/Ph.D. degree upon successful completion of their medical and research training. An original, in depth thesis research project is required.

The exceptional nature and continuing high quality of this unique NIH research training program has been recorded in an overwhelming consensus by the NIGMS formal peer review group and Advisory Council, senior NIH staff and consultant groups of the National Academy of Sciences. The record clearly shows that the combined degree candidates are truly outstanding, continue to hold their interest and keen ability in both fundamental research and clinical activities beyond the award of their doctoral degrees, and a high percentage of the earlier graduates are becoming established in research careers and competing well for tightening NIH research grant funds.

Twenty-three Medical Scientist Research Training Grants were awarded in Fiscal Year 1982, including a new grant to the University of Texas at Dallas, with provisions for support of 676 trainee positions. This is one less grant award than in Fiscal Year 1981 because support to two earlier programs was not continued with Fiscal Year 1982 funds, following peer review of their competing applications. Also, funds were limited to the support of trainees from all awarded grants to a maximum of six years. Consequently, the 23 grants in Fiscal Year 1982 provide for 29 fewer positions than last year. However, recommended increases are called for in future years, and other applications are under review for possible awards.

This well-established program valuably contributes to the entire NIH clinical and basic research manpower requirements by providing outstanding physician investigators. To assure its continued high priority and effective role within the Institute's major research training effort, a thorough assessment of the sharply increasing cost of this program, particularly in the tuition category, as well as its absolute dependency on the equally important regular predoctoral program of the Institute, was begun this year.

3. Basic Pathobiology Institutional Postdoctoral Training Program

The goal of this program area is to provide individuals holding a professional degree an indepth training experience in the principles and methods required for research at the cellular and molecular level in both normal and diseased states. In addition it also provides opportunities for a limited number of post-Ph.D.s to gain insight and develop experimental approaches to fundamental problems of human disease.

Some excellent research training opportunities are available. However, due to well-recognized factors, there has been a difficulty in attracting sufficient numbers of post-MDs into research careers via this route. As a result, the increased dialogue across the basic/clinical interface has been less than adequate. Applications from a few previously funded programs have not fared well upon re-review, and consequently, the number of trainee positions fell from 41 in Fiscal Year 1981 to 33 in Fiscal Year 1982. In order to strengthen their programs, other institutions have been attracting post MD/Ph.D. graduates of the Medical Scientist program who require a significant period of advanced postdoctoral research training to remain current and competitive for entering research careers beyond their residency speciality training. All of the trainees enrolled and recruited during Fiscal Year 1982 have excellent credentials and promise as developing scientists.

4. Cellular and Molecular Biology Individual Postdoctoral Fellowship Program

The Cellular and Molecular Biology Postdoctoral Fellowships provide individuals holding the Ph.D. or a professional doctoral degree an opportunity to acquire highly specialized research training in the most active research laboratories in the United States and abroad. The purpose of such training is to provide the most current state-of-the-art knowledge and concepts from leading investigators in a field immediatley prior to the young scientist establishing an independent research career. These opportunities and awards are highly selective in direct national competition.

The limited number of awards made this year does not reflect the number of high-quality fellowship applications being received. The unfortunate decline in awards during recent years is reflected in the following figures: 159 awards in fiscal Year 1980; 112 awards in Fiscal Year 1981; and only 69 awards in Fiscal Year 1982. This trend prevailed throughout the Institute and special attention is being given to reversing this decline in order to maintain an appropriate balance with the other NIGMS research training programs.

Research and Training Areas Supported by the Cellular and Molecular Basis of Disease Program

	No. of Grants*	Dollars (TC)
Cellular Basis of Disease Section		
Cell Regulation, Differentiation & Growth	85	7,234,000
Cell Organization, Motility & Division	86	7,897,000
Membrane Structure & Function	274	26,419 000
Section Total	445	41,551,000
Molecular Basis of Disease Section		
Enzyme Catalysis & Regulation	214	17,472,000
Glycoconjugates	24	2,396 000
Bioenergetics	72	7,132,000
Structural Studies of Proteins & Related Model Systems	146	12,789,000
Diffraction Analysis of Proteins & Related Model Systems	72	7,019,000
Theoretical Studies of Proteins & Related Model Systems	25	1,955,000
Section Total	553	48,765,000
TOTAL RESEARCH SUPPORT	998	90,316,000

RESEARCH TRAINING SUPPORT

Category	No. of Trainees	No. of Grants*	Dollars
Institutional Predoctoral			
Cellular & Molecular Biology	963	45	10,888,000
Medical Scientist	658	21	8,762,000
Total Predoctoral	1,621	66	19,650,000
Institutional Postdoctoral			
Basic Pathobiology	33	10	574,000
Individual Postdoctoral			
Cellular & Molecular Biology	67	67	1,124,000
Total Postdoctoral	100		1,698,000
TOTAL TRAINING SUPPORT	1,721		23,046,000

*active as of 6/30/82

RESEARCH PERSPECTIVES

Basic research is moving at a rapid pace in the areas funded by the Cellular and Molecular Basis of Disease Program (CMBD), as it is in many other areas such as those supported by the Genetics Program. Over the past decade, there has been a near revolution in the approaches and techniques used in molecular and cell biology. In many respects molecular biology has shown the way by revealing the power of the approach to higher processes through in vitro systems--especially using a detailed biochemical and biophysical approach. In addition, molecular biology has provided an intellectual framework for approaching questions in development, control, and cell maintenance. It also has demonstrated the value of applying genetic techniques in the attack on a wide range of biomedical questions.

Another major reason for the marked advances in the CMBD areas is the significant improvement in research instrumentation. There has been wide dissemination of sophisticated new techniques so that many problems are now routinely attacked simultaneously by several techniques involving advanced analytical instrumentation. However, over the past decade or two, the spread in instrumentation has been below what is truly needed for full use of these techniques. It has been difficult for individual users to have ready access to state-of-the-art instruments. Many first rate American laboratories have lagged behind those in Europe in this regard. However, the NIGMS Shared Instrumentation Program has made a good initial step towards alleviating the problem. Unfortunately, budgetary constraints make it impossible to fund all worthwhile facilities.

One trend, which is obvious but must be noted, is the remarkable spread of computers into the laboratories. This is due to several factors. First, computers have become more and more available and much less expensive. Microcomputers have brought the cost of off-line computation within the reach of any funded laboratory. Expansion of the memory capacity and software of these units has allowed investigators to buy them instead of minicomputers or lease time on large computers. "Home" computers are now available with 64 K byte memory and packages handling Pascal as well as FORTRAN languages. A second reason for the spread of computers is the increase in computer literacy. A new generation of graduate students has entered science. They possess a familiarity with computers and an eagerness to use this knowledge. Third, analysis of diffraction data has become more sophisticated. Modeling of interactions of systems, in fact, models as a whole have become more complicated and sophisticated. Data handling and "crunching" have been limited by the availability of storage capacity. The advent and availability of floppy discs have made data storage and processing on a large scale very much easier. Lastly, on-line, real-time analysis has progressed as computer speed has improved. In summary, it would be impossible to tour laboratories without seeing computers and terminals throughout. This would not have been true a decade ago. This availability has profoundly changed the way science handles masses of data or complex problems, and allows scientists to think of interactions and models in a way scarcely known a generation ago.

Some of the major advances in instrumentation are discussed under the specific "Highlights Section" below; but others deserve special mention. As an example, although protein purification methods have been continually improved, it remains difficult to purify many cellular proteins in quantities sufficient to sequence. This is especially true of hard-to-purify membrane proteins or phenotypic variants of common species. Sequencing is of special interest as a first step to understanding function--such as the configuration of binding or active sites, relationship to other proteins in a complex or lipid in bilayer, or determination of homologies with related proteins. At the same time, preparative separation techniques have improved, as have analytic ones. Sophisticated electrophoresis techniques in both one and two dimensions have been developed. As a result, a surprising variety of cellular proteins has been revealed. Unfortunately, it is not easy to use two dimensional electrophoresis as a preparative method, especially to isolate significant quantities of a protein.

The CMBD Program reported last year on the development of a new method of automated protein sequencing using gas phase reagents and a minute sample of protein. This method, developed by a team of investigators at California Institute of Technology, with NIGMS support, can give sequence data on as little as 5 pmol of protein and can determine sequences of more than 30 residues on subnanomole quantities purified by SDS gel electrophoresis. The developers are still improving the instrument and coupling it with a mass spectrometer to allow sequencing of samples down, perhaps, to the fmol range. Although this microsequinator is unique, a version is being developed for commercial production. At present, only a few proteins or peptides have been sequenced with this instrument, but when commercially available, the impact on the field will be profound.

How does one obtain sufficient quantities of a protein to purify and study? The two major advances in identification and synthesis of proteins are the development of hybridoma and recombinant DNA technologies. It is difficult to overestimate the impact that these technologies have had on biomedical research. We have long possessed the ability to raise antibodies against cellular proteins. Such antibodies have been used in diverse ways--as selective agents when attached to an affinity column, as specific histochemical reagents, and as reasonably specific identifiers of homologous proteins. Although polyclonal rabbit and goat antiboides have been used by many investigators for several decades, in a sense they were underutilized. Researchers who might have been able to make good use of polyclonal antibodies were often unfamiliar with the techniques or unaware of the potential value. The development of mouse hybridoma technology, by which highly specific antibodies are produced by cultures derived from cloning a single lymphocyte, has done more than just provide an incredibly useful tool in cell research. It has provided excitement to a field and made everyone aware of the multitude of uses of such a wonderful natural reagent--one with enormous specificity--not just to a specific peptide but often a specific region of the peptide.

Although the NIGMS is divided into several programs, including the Genetics Program and the Cellular and Molecular Basis of Disease Program, the divisions are arbitrary. More than that, however, despite the wide range of modern biomedical science, techniques, approaches, and concepts developed in one area spread quickly to others. An outstanding example is the use of recombinant DNA technology. This technology allows investigators to isolate genes, clone them, and induce them to synthesize their products in large quantities. Although many of the techniques involved are well-established and almost routine, it still is a formidable task to complete the entire procedure; that is, isolate the desired gene and proceed to the point of synthesis of significant quantities of the pure product. Nevertheless, success at even part of this procedure can yield significant benefits. For example, increasing the copies of a gene coding for a membrane protein, even if the gene has not been fully isolated, may allow study of the role of the protein in the membrane, or make isolation of monoclonal antibody that much easier. This array of potent techniques has now become an integral part of cell biology. In the past year or two, genes have been cloned which code for membrane receptors, tubulin, cytochromes, and much more.

The array of new techniques which can: 1) increase the quantity of material to be studied; 2) improve the purity and yield of purification; and 3) increase the sensitivity of the measurements to be made on these proteins, all combine to multiply many fold the proteins which can be studied. As was pointed out last year, many developments may occur gradually, but the consequences may be a seemingly sudden opening up of new vistas.

Techniques developed with support in the CMBD Program are also proving of use in other areas. For example, this program supports a large fraction of the domestic research on X-ray crystallography as well as other related techniques. These techniques are now being used to look at nucleotides and chromosomal structure as well as the structure of nucleotide-protein complexes. The electron diffraction techniques originally developed for structural analysis of membrane proteins is being used to study ribosomal substructure. The membrane reconstitution techniques discussed in the "Highlights Section" below are utilized extensively in the study of membrane receptors of pharmacological interest. Lastly, the nuclear magnetic resonance technology, developed in part for the study of physical chemistry and biochemistry, is being expanded to look at all types of systems over a wide range of dimensions. This same biochemical/biophysical technology now promises to provide in vivo pictures of soft tissues of organs. Such pictures surpass any others obtained from the several other radiologic techniques in current or projected use.

In the 1981 Annual Report, we discussed the extensive support the Institute gives to studies of the role of intracellular calcium and its mode of entry into cells. We underlined the support of research on calmodulin and the increasing interest this work has generated. If anything, the interest in calmodulin has increased over the past year. Also increasing has been research into the control of calcium entry. Several years ago calcium ionophores--that is, compounds which facilitate the transport of calcium across membranes--were discovered and the mode of such ion selectivity probed. The selectivity and mechanism of native ion channels has also been of intense interest. One highlight on the work of Dr. Olaf Andersen and colleagues on a similar, potassium-selective ionophore, is included below. Following the discovery of natural calcium-selective channels, many biophysicists looked for compounds which would selectively block these channels. The parallel was drawn to tetrodotoxin, a selective blocker of a class of sodium-selective channels, whose use has resulted in an immense increase in our knowledge of sodium-selective and other channels. An intense effort has revealed a few which are calcium-channel blockers. The first one which was reasonably selective and effective as a blocker is called verapramil. Subsequently, other "calcium blockers" have been found. This class of drugs now appears to be effective against the pain of angina. Verapramil and at least one other blocker have been approved by the Food and Drug Administration for use in treatment. These blockers hold out considerable promise against a debilitating illness. It has been known that cardiac and smooth muscle cell let in calcium ions during electrical activity, and that ion can enhance the contractions. This is another example of the broad reach of research into basic cellular processes.

RESEARCH HIGHLIGHTS

Presented below are examples of recent accomplishments from a variety of cell and molecular systems being investigated by grantees of the Cellular and Molecular Basis of Disease Program. They were selected to reflect the broad range of activities currently being supported. They also reflect the far-reaching importance of individual lines of research and the remarkable spread of the research across conventional subject boundaries. Thus, the work of Fridovich, although originally concerned with the structure of an enzyme, has subsequently revealed surprising findings of interest to investigators concerned with molecular biology or evolution. The work of Sturtevant, using the physical technique he helped develop, is now addressing a fundamental question about the control of a process of interest to molecular biology. Staehelin, using the techniques of electron microscopy and biochemistry, is posing questions of central interest to both anatomists and biochemists. De Rosier, a noted X-ray crystallographer, has collaborated with Tilney, a distinguished cell biologist. Together they have proposed a model which could explain the connection between ATP hydrolysis and cell motion. Nearly all the highlights are of similarly broad import. Collectively, they demonstrate the tight intermeshing and interdependence of all areas of basic biomedical research.

"Monte Carlo Simulation of Water in Biological Systems"

R01 GM 30793-01 (Hagler, A.), Agouron Institute, University of California, San Diego

In this work, Dr. Arnold Hagler and his colleagues are studying the aqueous environment of biological molecules by theoretical computer simulation techniques. The solvent system which constitutes the environment of biological macromolecules, such as proteins and nucleic acids, plays a central role in both their structure and function. In spite of this crucial importance, relatively little is known about the structure and properties of this aqueous environment, either from an experimental or theoretical vantage point, due to the inherent difficulties associated with the study of the liquid state in general, and liquid water in particular. These difficulties exist in both experimental and theoretical descriptions, and arise from the nature of the "configurational entropy" or inherent disorder of the liquid state.

In order to attack this problem, Dr. Hagler has extended the "Monte Carlo" method to the simulation of water structure around molecules of biological interest in crystals and in solution. The Monte Carlo method and the related method of molecular dynamics are extremely powerful theoretical techniques developed to study liquid structure. They are statistical mechanical methods, based on the fundamental intermolecular and interatomic forces and energies of the system. The advent of the current generation of powerful computers, along with the development by Dr. Hagler of software for energy surface calculations for proteins, has allowed him to extend these methods to the study of the even more complex biological systems.

For the initial study of the aqueous environment of a protein by these theoretical techniques, he chose to simulate the water structure about the enzyme lysozyme in its triclinic crystal. The computer simulation of a crystal system in which there are many waters of hydration has several advantages, not least of which is the opportunity for comparison with experimental data. For example, lysozyme contains 129 amino acids and the unit cell of the triclinic crystal contains 303 water molecules. When neighboring unit cells are included, the system to be simulated includes about 8000 protein atoms and 2000 water molecules. Several interesting questions can then be addressed: What is the nature of water "bound" to the protein? How much "bound" water is there? What is the nature of the solvent in the first hydration layer of the protein? How far away from the protein does the solvent still feel the protein's influence?

Even at this preliminary stage, several interesting observations were made. Water molecules in the protein crystal are found to vary from fully disordered with energy distributions similar to that of bulk (pure) water for molecules far from the protein surface, to highly ordered water molecules with low average energies, close to charged groups on the protein. This range of order is in good agreement with that observed crystallographically. The first hydration layer, or water molecules in intimate contact with the protein, was found to be heterogeneous rather than a homogeneous coat. Thus, the properties of the water molecules, their energies and degree of order, depend, not surprisingly, on the particular amino acid with which they interact. Thus, it appears one cannot speak simply of a "bound" layer of water. Properties of water molecules lying in the substrate binding cleft were also examined. These water molecules are an important factor in the energetics of substrate binding. An interesting finding here is that, even in apparently favorable polar and sometimes charged environments, the detailed geometry of the protein makes it impossible for several waters to orient themselves so as to obtain optimum interaction energies. Thus, the hydration of the cleft may be less favorable than would be thought simply on the basis of the hydration of the individual amino acids constituting these sites.

In addition to the study of water about lysozyme, Dr. Hagler has also undertaken a detailed study of a crystal system containing two cyclic hexapeptides and sixteen waters per unit cell. This study was undertaken for two reasons. First, hydration of small peptides has become an increasingly important field in relation to the understanding of the conformational behavior of small biologically important molecules such as peptide hormones. Second, the small size of this system and the accurate X-ray model make it an ideal benchmark for testing the methods and intermolecular energy functions. This was the first application of a "Monte Carlo" simulation of water-peptide interactions, and water structure in a peptide crystal. The water structure was characterized in terms of the statistically averaged positions of the individual molecules, their (root-mean-square) movements about these positions, and examination of individual configurations. In addition, the variation in water structure in various peptide environments was studied and correlated with the energetics of the individual water molecules.

Other work in this laboratory focuses on the effect of solvent on the conformational equilibrium of a small peptide in solution. The solvent-peptide interaction was shown to perturb strongly the balance between an extended and helical conformation, favoring the latter significantly.

The significance of this work lies in the ability of these theoretical techniques to give a clear understanding of the water structure, the hydrogen bonding networks, and the fluctuations in these structural features, to a degree hitherto unavailable from either experimental or theoretical studies. Dr. Hagler has begun to understand biological systems at the molecular level in terms of the fundamental energetics and dynamics of the system.

Dr. Hagler plans to extend this work to other small peptide crystal systems to provide further insight and tests on well characterized systems; to the study of such peptide secondary structures as helix and extended strand in aqueous solution; to an additional protein system; and to further study of substrate and inhibitor binding.

"Development of Scanning Calorimetry"
R01 GM 04725-26 (Sturtevant, J.), Yale University

Two distinct types of calorimetry (the measurement of heat produced in a chemical reaction) are finding wide application in biochemistry. The older of these, isothermal calorimetry, is employed in the straightforward determination of enthalpy changes, and in some cases of equilibrium and kinetic data. A newer type of calorimetry, differential scanning calorimetry (DSC), is proving to be an extraordinarily powerful technique. In DSC, the process under study is initiated by changing, usually increasing, the temperature. The temperature-dependent transitions which can be observed provide a measure of enthalpy and heat capacity changes accompanying chemical processes.

Dr. Julian Sturtevant, Professor Emeritus at Yale University, is a recognized leader in the field of calorimetry. He and his associates are using this technique to determine thermodynamic properties of a wide range of biological systems. His current research involves the application of DSC to evaluate the domain structure of proteins. Thus, Dr. Sturtevant has collaborated with Drs. Pabo, Sauer, and Ptashne of Harvard on a study of the λ repressor protein, showing that it contains two independent denaturable domains. Papain digestion of this protein produces two segments that are relatively resistant to further digestion. DSC showed a denaturation transition at 50°C for the amino-terminal fragment, and 70°C for the carboxyl-terminus. Intact repressor shows two transitions, one at 50°C and one at 70°C. These and other data show that λ repressor contains two domains connected by a protease-sensitive link. One domain oligomerizes and the other binds DNA.

In his research, Dr. Strutevant applies physiochemical techniques and principles to a wide variety of biochemical problems.

"Structural Kinetic Studies by Nuclear Magnetic Resonance"
R01 GM 20168-10 (Redfield, A.), Brandeis University

The nuclear magnetic resonance spectrum of transfer RNA (tRNA) provides useful indicators of conformation and dynamics. The imino protons are especially interesting because the rates of their exchange with solvent can be measured by NMR to obtain information on structural mobility. However, the problem of assigning resonances has proved formidable. Of the groups active in this field, by far the most innovative has been Dr. Alfred Redfield's. Methods developed by Dr. Redfield have proven highly successful in unraveling structure, as have the improvements in instrumentation, also developed by his group.

A major tool used by Dr. Redfield to map protons that are adjacent in the folded tRNA is the nuclear Overhauser effect (NOE). In this approach, a weak preirradiation pulse is applied at the frequency of a specific proton. Adjacent proton lines will be altered in intensity as a consequence of the pre-saturation of the target proton. This has allowed Dr. Redfield to assign more NMR lines than any other competing method, and also to correct mistaken assignments by other groups. Using these assignments, he has been able to obtain for tRNA a more detailed picture of thermal unfolding processes than for any other macromolecule. Most of the higher temperature rates reflect unfolding of major parts of the molecule, in agreement with optical temperature-jump relaxation studies. A detailed picture has been established for yeast $tRNA^{Phe}$. The first event, at 33°C, is a single end-fraying of the GC1 base pair. At slightly higher temperatures, some tertiary resonances start to melt out. Unfolding of specific tertiary structures can be followed up to 40°C, the highest temperature measured. Secondary structure begins to melt in the acceptor stem. This is particularly interesting, since this is a relatively long helix. After that comes the D stem, followed by the anticodon and T stems.

These measurements provide evidence for structural stability and structural mobility of a macromolecule whose function may be critically related to changes in conformation.

"Crystal Structure of *Azotobacter vinelandii*"

R01 GM 25672-04 (Stout, C.), University of Pittsburgh

Dr. Charles Stout and his coworkers are studying several electron transfer protein molecules from *Azotobacter vinelandii*, a bacterium that reduces both atmospheric oxygen and nitrogen. The goal of this project is to study the metabolism of the cell at the basic level of molecular structure using the technique of X-ray diffraction. This work should lead to an understanding of the interface between several proteins involved in electron transport in the same organism. Five electron transfer proteins have been isolated and purified from this bacterium: two iron-sulphur (Fe-S) proteins, two cytochromes, and one flavin protein. In the initial project, Dr. Stout recently solved the structure of one of the Fe-S proteins, ferredoxin.

This molecule was crystallized in four polymorphs. The tetragonal form with one molecule in the crystallographic asymmetric unit was chosen for the initial experiments. Electron density maps at 2.5 Å resolution based on a single heavy atom derivative and at 3.0 Å resolution based on three derivatives have permitted Dr. Stout to determine the structure of the amino acid chain and the Fe-S clusters. This structure has provided a real surprise. The protein was found to contain two different Fe-S clusters with a total of seven Fe atoms. Structures were modeled as $[3Fe\text{-}3S](S\lambda)_5$ and $[4Fe\text{-}4S](S\lambda)_4$, where S represents cysteine sulfur atoms of the protein. Previously recognized types of Fe-S clusters are $[4Fe\text{-}4S](S\lambda)_4$, $[2Fe\text{-}2S](S\lambda)_4$ and $Fe(S\lambda)_4$. Refinement of the complete protein structure at 2.0 Å resolution confirmed the assignment of protein ligands: *Azotobacter* ferredoxin contains Fe-S clusters of composition $[4Fe\text{-}4S](S\lambda)_4$ with cysteines 24, 39, 42 and 45 and $[3Fe\text{-}3S](S\lambda)_5$(Oxo) with cysteines 8, 11, 16, 20, 49, and Oxo, a non-protein ligand--most probably a water or hydroxyl molecule.

The use of 9 cysteine ligands by this 7Fe-ferredoxin to make the two Fe-S clusters follows a pattern fundamentally like that in 8Fe, 8 cysteine containing bacterial ferredoxins, but the protein structure differs radically from the known representative 8Fe-ferredoxin structure. Two extra residues between the second and third cysteines, and a pentapeptide between the third and fourth, allow the N-terminal segment of the Azotobacter protein to fold around the 3Fe center. The unique positioning of the extra (9th) cysteine allows the 4Fe center to be formed in such a way that its local stereochemistry differs from 4Fe centers of the 8Fe-ferredoxin. Sequence homologies of the Azotobacter 7Fe-ferredoxin and several ferredoxins from other species suggest that 7Fe-ferredoxins folded into a similar conformation are potentially widespread in occurrence.

The 7Fe-ferredoxin molecule contains 106 residues (compared to about 54 residues for 8Fe-ferredoxins), but the last cysteine is only the 49th in the sequence (J. B. Howard). The additional protein is important in creating an unsymmetrical charge distribution. The environment of the 4Fe center is strongly acidic (15 glutamates and 10 aspartates); the region of the 3Fe center is overall close to neutrality with 6 basic residues (lysine, arginine, histidine) and 6 acidic residues (glutamate, aspartate and caboxy-terminus). A third domain of the molecule is comprised of a large, open loop (residues 80-90) which forms a cleft leading to the Oxo atom on the [3Fe-3S] cluster. This region of the structure contains 9 negative and 3 positive charges. The molecule has a high net charge and is therefore highly hydrated in the crystal. Thus far 344 of a total of 740 waters per asymmetric unit have been refined. The water structure extends into the cleft region and is in contact with Oxo and a sequence conserved glutamate (residue 18) that is adjacent to the 3Fe center.

The protein structure exhibits very little regular secondary structure, except for short regions of 2_7 helix and one anti-parallel β loop. There are a number of β turns contributing to the Fe-S cluster coordination and at least 26 intramolecular hydrogen bonds. Features of the 4Fe cluster include two aromatic residues adjacent to the 4Fe center, 4 hydrogen bonds to its sulfur atoms from the protein, and 4 water molecules in contact with the cluster. The bond distances and angles of the [4Fe-4S] cluster are most like those that have been observed for the $[4Fe-4S]^{2+}$ cluster in reduced high-potential iron protein. There are no aromatic amino acids in the vicinity of the 3Fe center and only 3 hydrogen bonds to this low-potential cluster. The sulfur atoms of $[3Fe-3S](S_y)_5(Oxo)$ are in van der Waals contact with peptide plane atoms and carbonyls of the protein. In addition, there are five potentially short contacts between sulfur atoms. The bond distances and angles for the [3Fe-3S] cluster show that the internal ring angles of inorganic sulphur are anomalously large, and that the coordination around each Fe is distorted markedly from tetrahedral. The Fe•••Fe separation, 4.1 Å, is distinctly greater than in 4Fe centers (about 2.8 A) and the [3Fe-3S] ring is nearly planar (displacements of sulfur from the plane of the three Fe atoms no more than 0.5 Å). In addition to the Oxo atom, the 3Fe center and glutamate 18 are in contact with 6 water molecules.

Although only one three-iron structure has thus far been solved, spectroscopic results from a number of investigators have identified three-iron clusters in several other proteins. The three-iron cluster appears to perform a variety of functions in different proteins. Their results show that the cluster is highly sensitive to the surrounding protein conformation, and is, in fact, able to undergo change, both in the conformation and in ligand binding.

Work in Dr. Stout's laboratory on the other Azotobacter electron transport proteins is proceeding. Another Fe-S protein, aconitase, contains a cluster with properties related to [3Fe-3S] clusters. This enzyme catalyzes the stereospecific conversion of citrate into isocitrate. Good quality crystals have been grown and a structure determination is underway. He has also started work on the structure of Azotobacter cytochrome c_5, which is a diheme protein. Four different crystal forms have been obtained. The flavodoxin has an unusually stable semi-quinone state and contains two phosphates per molecule in addition to the flavin mononucleotide. Crystals in two space groups have been found for this protein.

"Ligand-Bridged Binuclear Metal Complexes and Proteins"

R01 GM 16449-14 (Lippard, S.), Columbia University

Superoxide dismutase (SOD) has been studied extensively. This protein provides protection for aerobic organisms and tissues against free radical damage from the superoxide radical (O_2^-). This occurs by the conversion of the radical to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). The Cu-Zn superoxide dismutase is found in the cytoplasm of a wide variety of eukaryotes. The enzyme isolated from bovine erythrocytes is composed of two identical subunits, each of which contains one copper (II) and one zinc (II) ion. One of the ligands is a histidine imidazole which bridges the copper and zinc. Such ligand bridged bimetallic centers are widespread in biology. In SOD, the reduction of the superoxide ion is known to occur through oxidation-reduction of the copper. What, however, is the function of the zinc? Studies by Dr. Stephen Lippard have demonstrated that zinc was important in organizing the active site structure. It was also suggested that breaking of the imidazole bridge was involved in the mechanism of SOD action.

A derivative of SOD has been produced in which the zinc has been replaced with a second copper (Cu_2Cu_2SOD). Low-temperature ESR spectra of this derivative can easily distinguish the bridged enzymes from one in which the coppers are magnetically isolated. When thiocyanate is added, the imidazole bridge is broken. The SCN is liganded directly to the Cu^{2+} in the copper site, and replaces the aspartic acid on the Cu^{2+} in the zinc site. On the zinc side, therefore, there is an imidazole-Cu^{2+}-SCN linkage. Since this complex is catalytically active, breakage of the bridging imidazole group cannot be essential to the enzyme mechanism. Controls performed with Ag_2Cu_2 SOD in the presence of SCN showed that the activity was not due to the copper in the zinc site.

The function of the zinc in SOD thus seems to be primarily, if not yet exclusively, in forming the active site. Since SOD is the only copper-protein with an X-ray structure determined to 3Å, these studies provide a unique opportunity to analyze the function of bridged bimetallic centers.

"Gene Transfer Across the Eukaryotic: Prokaryotic Abyss"

R01 GM 10287-20 (Fridovich, I.), Duke University

Superoxide dismutases are enzymes which convert the very reactive superoxide free radical (O_2) to hydrogen peroxide. Since it is now known that the superoxide radical is a commonplace cellular product of oxygen reduction and an important cause of oxygen toxicity, the peroxide dismutases provide an essential defense against the reactive free radical for aerobic organisms.

The superoxide dismutases can be divided into three major groups. The first of these contains copper and zinc, the second contains manganese, and the third contains iron. Amino acid sequence data indicate that the iron and the manganese enzymes derive from a common ancestral protein, while the copper-zinc enzymes represent an independent line of descent. While the copper-zinc superoxide dismutase is characteristic of the cytosol of eukaryotes, the iron enzyme is found in prokaryotes and the manganese enzyme is found in prokaryotes and the mitochondria of eukaryotes.

Since this distribution of these types of superoxide dismutase is well-established, it came as a surprise when a French laboratory in 1974 reported the existence of a copper-zinc enzyme in the bioluminescent bacterium Photobacter leiognathi. This anomaly has been addressed by Dr. Irwin Fridovich of Duke University, who has studied superoxide dismutase for many years with NIGMS support (GM-10287-19). In 1975, Dr. Fridovich suggested that since P. leiognathi lives as a symbiont in the ponyfish, the bacteria may have acquired the gene, which codes for the copper-zinc superoxide dismutase, from the fish. Dr. Fridovich and his co-worker, Dr. Joseph Martin, have now published evidence which substantiates this idea. The evidence consists of a statistical comparison of superoxide dismutase amino acid compositions, as well as a study of the physical and chemical properties of the copper-zinc enzymes from the ponyfish and P. leiognathi.

In the statistical study, a comparison, seeking similarity, was made of the amino acid compositions of 59 different superoxide dismutases from diverse biological sources, including those from the ponyfish and P. leiognathi; this comparison correctly grouped the copper-zinc, manganese, and iron enzymes. When another statistical analysis was applied to the group of copper-zinc superoxide dismutases, three subgroups were recognized. One of these was composed of the enzymes isolated from birds and mammals. The second was the group of enzymes from plants and fungi, and the third consisted of the enzymes from fish and from P. leiognathi.

Biochemical evidence also indicated the close similarity of the enzymes from P. leiognathi and those of ponyfish and other teleosts. Determinations of molecular weight, isoelectric point, subunit characteristics, optical spectra, and sensitivity to chemical inactivation all were consistent with the near identity of ponyfish and P. leiognathi copper-zinc superoxide dismutase. Data on the amino acid sequence of the two proteins have not yet become available.

These studies, coupled with the absence of a copper-zinc superoxide dismutase in any of some 95 other strains of marine and terrestrial enterobacteria which have been examined, and the long and intimate symbiotic relationship which has existed with these two organisms, have lead Drs. Fridovich and Martin to conclude that a gene transfer likely occurred from the ponyfish to P. leiognathi. They have forthrightly pointed out that there is no known role for the copper-zinc superoxide dismutase in P. leiognathi that could have caused this gene to be retained by the bacteria. Nonetheless, these studies do suggest that the eukaryotic genome may be less rigidly excluded from naturally occurring recombinant DNA technology than previously assumed.

"High Technology Leads to Important Progress in Bioenergetics"

R01 GM 27308-04 (Chance, B.), University of Pennsylvania

A most important process for the maintenance of life is the process whereby the energy contained in foodstuffs is converted to ATP, with concomitant oxygen consumption. Deprivation of oxygen is a rapid and deadly killer through cardiac arrest or catastrophic stroke. Likewise, toxic gases that mimic oxygen or certain environmental poisons that can block oxygen delivery or use in the tissues leave the body in a de-energized state.

The final step in the formation of ATP involves the transfer of electrons to cytochrome oxidase, an enzyme located in the mitochondrial membrane. Cytochrome oxidase contains iron and copper atoms that are capable of reacting specifically with oxygen and reducing it to water. The study of this unique reaction has not been possible by conventional biochemical techniques because the effects of the two metal atoms upon each other obscure any measurements of the interaction of the metals with oxygen. In order to study the interaction of the metals with the cytochrome oxidase enzyme surrounding them and to measure the distance between the metals, Drs. Britton Chance and Linda Powers, supported by grants GM 27308 and GM 27476, have developed instruments permitting the use of very high intensity X-ray sources for the study of the very small amounts of the copper and iron present in cytochrome oxidase. Intense X-ray beams are available from the high energy sources used by nuclear physicists at the Stanford Synchrotron, and soon an even higher intensity beam will be available at the Brookhaven National Light Source on Long Island. These intense X-ray beams elicit radiation from the iron and copper atoms in cytochrome oxidase which is then reflected from nearby atoms. Measurement of these reflections has permitted for the first time a direct study of the interactions of the iron and copper atoms and the changes in their relationship to oxygen in the functioning enzyme. This technology has provided, in only a few years, structural information that the classical methodology, X-ray crystallography, has not been able to produce.

Drs. Chance and Powers have utilized the synchrotron methodology to obtain two striking and unexpected results that unify and greatly extend seemingly diverse ideas on oxygen binding by hemoglobin and on plant and yeast enzymes (peroxidases) that metabolize hydrogen peroxide.

The reactions of cytochrome oxidase revealed by the synchrotron method are of great interest. Starting with the reduced form, the iron-copper couple combines with oxygen in a reaction which, on a structural basis, appears to be very similar if not identical to that of hemoglobin with oxygen. Electrons are transferred from the iron and copper atoms to oxygen, reducing it to peroxide, binding and bridging the peroxide between the iron and copper atoms appropriately spaced at a distance of 3.8 Å. Thus the toxic and reactive molecule, H_2O_2, is maintained safely bound between the correctly spaced iron and copper atom. Further addition of electrons and protons reduces the peroxide to water and generates the oxidized forms of iron and copper in a non-bridged oxidized form which can react with peroxide compounds in this manner similar to the peroxidases. Two of the forms of the enzyme resemble the peroxidases from yeast and plants in their structures and reactivities. A novel form of the enzymes is obtained when the sulfur atom attached to the copper bridges between the iron and copper with a remarkably long Fe-S bond of 2.6 Å.

The presence of iron in cytochrome oxidase was first observed by Dr. Otto Warburg in 1929 and its chemical properties were suggested to be vastly different from any known iron compound, particularly different from that of the oxygen-transporting iron atom of hemoglobin in blood cells. The results from the synchrotron studies show that the ideas of Dr. Warburg and those who followed in the succeeding 60 years need to be modified. The structural properties of the iron atom of reduced cytochrome oxidase iron are identical within 0.02 Å to those of reduced hemoglobin itself. This very special structure allows hemoglobin and reduced cytochrome oxidase to combine extremely rapidly with oxygen for their respective functions of transport and oxygen reduction. The similarity of these two structures suggests that there is an optimal, unique structure for rapid binding of oxygen.

The structural similarities between the form of cytochrome oxidase that lacks the above mentioned iron-sulfur-copper bridge and the structure of peroxidase from plants and animals is remarkable. Here again, the structural similarity suggests an optimum for reactions with peroxide as found in peroxidases and the oxidized cytochrome oxidase. In this case the iron atom reacts avidly with the toxic chemical hydrogen peroxide, preventing it from escaping and damaging delicate cell constituents.

In these two cases the great bulk of structural, kinetic, and mechanistic information on oxygen transport proteins of the blood such as hemoglobin and on peroxidases of plants and animals now becomes applicable to the study of cytochrome oxidase. Furthermore, these discoveries afford a common structural ground for the two important functions of cytochrome oxidase, namely oxygen binding and peroxide binding, thereby unifying two diverse fields of research, oxygen transport and cell respiration.

"Let the Sunshine in on Bioenergetic Membranes"
R01 GM 12202-19 (Mueller, P.), University of Pennsylvania

In animal and plant cells, energy conversion takes place in the membranes of mitochondria and chloroplasts. Their membranes are composed of a lipid bilayer with special proteins extending through it from one side to the other. The first step in the energy conversion process is the movement of electrons. High energy electrons derived from the chemical bonds of food or sunlight are transferred through the protein across the bilayer generating an electrical potential and a proton gradient which are then used to form high energy compounds such as ATP. Neither water nor lipids can conduct electrons, but specialized proteins with metalloorganic components allow electron conduction through their interior. There are several different electron transfer proteins -- cytochrome oxidase, cytochrome b-c_1 and photosynthetic reaction centers, to name only a few.

The mechanism of electron transfer has been the focus of a major portion of bioenergetic research. Dr. Paul Mueller, supported by a program project grant to Dr. David Wilson (GM 12202-18), has been exploring the function of electron transfer proteins by reassembling the isolated components of bioenergetic membranes in artificial membrane bilayers designed to allow measurements of electron flow not previously possible.

It is very difficult, with present technology, to measure the currents and potential from these small organelles. However, Dr. Mueller has been able to construct planar lipid bilayer membranes which separate two compartments large enough to allow measurement of electrical currents directly. Into the lipid membranes, Dr. Mueller incorporated electron transfer proteins such as cytochrome oxidase and photosynthetic reaction centers (RC) and for the first time directly measured the currents and potentials generated by electron transport.

The reaction centers are particularly suited for such studies because the electron transfer can be initiated by a very brief flash of light allowing center kinetic measurements of the electron movement after the flash. The reconstituted membranes contain appoximately 2.5×10^9 reaction centers/cm^2. The amplitude of the flash-induced potential change is commensurate with the membrane capacity and the integral of the current due to a single flash. Each center can transfer about 5×10^9 electrons per second, and with about 2×10^{11} centers per cm^2 the membrane can reach current densities of about 150 A/cm^2 in response to brief intense flashes of light, although under physiological conditions the electron transfer rates are much slower.

By measuring the currents and potentials under different experimental conditions, Dr. Mueller was able to show which of these steps generate a current in the direction normal to the membrane plane and thus contribute to the potential. The electron transition from the chlorophyll to the primary quinone generates approximately 60% of the total current. The remainder results from the subsequent electron transfer between cytochrome c and the chlorophyll. The transition between the primary and secondary quinone and the quinone pool is not electrogenic, that is, does not contribute to the electrical potential. However, some of the pool quinones can diffuse across the membrane as anions and cause a small reverse current. In addition, the membrane potential can be held constant at different values to determine how the potential modifies the electron transfer and membrane currents. Within the potential range that is normally generated by the membrane--a few tenths of a volt only--the electron transition between cytochrome c and the chlorophyll is affected, but not the subsequent reactions. Thus, under normal conditions, the membrane potential generated by the electron transfer can in turn control the electron flow; and it does this at the cytochrome c level.

The currents initiated by a flash of light or a longer period of illumination have a rather complex time course, because the electrons pass through a number of steps. Nevertheless, it is possible to account quantitatively for all their details by calculating the rates of electron transfer between the different donors and acceptors.

In the natural photosynthetic membrane, the electron returns from the quinone together with a proton to the cytochrome c. This return is mediated by cytochrome b-c_1 another electron transfer protein which is also present in mitochondria. Electron and proton transfer through the b-c_1 complex, the cyclic electron transport initiated by light can be studied in greater detail than previously possible.

The lipid and water environment of the reaction centers (RC) in reconstituted bilayers still pose certain experiment restrictions. In order to overcome these, Dr. Mueller has deposited single oriented monomolecular layers of pure reaction centers from an air-water interface onto electrically conducting glass slides and evaporated a layer of metal on top of the RC. These sandwiched RC layers not only generate much larger currents and potentials, but also allow measurements down to liquid helium temperature for the study of electron tunnelling phenomena. In addition they permit spectroscopic analysis revealing the orientation of the chlorophyll and because of the lower access resistance to the RC, provide much improved time resolution of the electrical measurements. By depositing the RC at an air-water interface, he was able to obtain planar RC crystals, suitable for image analysis, which should ultimately reveal the structural details that must be known before the mechanism of energy conversion can be fully understood.

Aside from their immediate, obvious relevance in the field of cell function, studies of biological electron transfer have implications in other areas, as well. The electron transfer components are precisely aligned within the protein and their energy levels are exactly adjusted for optimum performance. All the complex functions are performed by a machine of molecular dimension, and insights gained from their study may lead, in the hands of physicists and engineers, to the construction of more efficient energy conversion devices, room temperature organic superconductors, microelectronic circuits and perhaps even molecular computing circuitry.

"Membrane Adhesion in Chloroplasts"
R01 GM 22912-06 (Staehelin, L. A.), University of Colorado

Photosynthesis, as carried out by oxygen-evolving organisms, involves two photosystems (PS I and PS II) that function in series. Electrons derived from the photolysis of water first pass through PS II, then through PS I. Maximal efficiency of electron flow requires PS I and PS II to turn over at equal rates. If one photosystem is excited more than the other, the excess excitation energy is dissipated with a loss of efficiency. Over a decade ago it was discovered that the optimization of this electron flow is achieved by regulating the distribution of absorbed excitation energy to the two reaction centers. However, the mechanism of regulation of energy distribution has eluded the sustained efforts of many investigators.

Recently, several laboratories have reported that the regulation of energy distribution between PS I and PS II is mediated by the phosphorylation/ dephosphorylation of an abundant chlorphyll-binding protein chl a/b LHC. Light induced phosphorylation of this protein increases the transfer of excitation energy from PS II to PS I. Dark induced dephosphorylation has the reverse effect.

Dr. L. Andrew Staehelin and his collaborators have found that this phosphorylation produces very pronounced and reversible structural rearrangements within chloroplast membranes. Chloroplast membranes are differentiated into stacked (grana) and unstacked (stroma) membrane regions enriched in PS II and PS I respectively. Several years ago Dr. Staehelin showed that chl a/b LHC was responsible for membrane adhesion. He has now shown that phosphorylation of chl a/b LHC reversibly reduces the membrane stacking. This unstacking allows about 30 percent of this protein to leave the grana and migrate up to 0.3 nm to the stroma. Regulation of the activity of the two photosystems is dependent on the spatial organization of chloroplast membranes at the supramolecular level. Although the regulation of interaction between two membranes by movement observable in an electron microscope has not been seen previously, it is unlikely that this is a unique system. It should alert us to look for structural correlates of other biochemical events.

"The Permeability of the Gramicidin A Channel"

R01 GM 21342-09 (Anderson, O.), Cornell University

Until 15 years ago, the terms "carrier" and "pore" were used only as hypothetical constructs, often best left to be cartooned in the "Discussion" section of papers. Now, due to the work of many investigators, starting with NIGMS grantee P. Mueller and including grantees D. Tosteson, A. Finkelstein, G. Leavitt, D. Urry and G. Eisenman, it has become apparent that the antibiotic gramicidin A functions as a pore or channel-former. The compelling evidence that it forms channels is the discrete conductance steps of uniform size seen in membranes treated with very small amounts of gramicidin A. The magnitude of these steps precludes any carrier mechanism. It is now confidently felt that the transport of most ions and polar electrolytes occurs through proteinaceous pores spanning cell membranes. This has made the study of the mechanism of pore formation and transport through gramicidin A channels all the more exciting. Gramicidin A has a known, relatively simple structure, and a reasonable molecular model of the channel exists. The channel exhibits ideal cationic selectivity--in fact its selectivity resembles that of many naturally occurring membrane channels. Lastly, the molecule is available in large quantities and can be chemically modified.

Many investigators have turned their attention to the conformation of the actual channel. There is substantial evidence that the channel is a hydrogen-bonded head-to-head helical dimer which spans the bilayer. According to the most accepted model, the polar groups of the peptide line the lumen of the channel, while the hydrophobic sidechains form the exterior surface, stabilizing the dimer in the membrane. The gramicidin A channel is thus a very narrow structure, consisting of an array of coordination sites associated with the polar head groups (carbonyl oxygens) of the peptide backbone. An ion or water molecule would have to move through this array by a series of rapid jumps from site to site.

Dr. Olaf Andersen and others have investigated the biophysics of transport of an ion through this channel and across the membrane. By a mixture of clever electrical biophysical measurements and careful and exhaustive theoretical analysis, he has concluded that the transport of ions and water through most of the length of the channel is by a single file process; that is, cations and water cannot pass each other. The rate of translocation is limited by the necessity for an ion to move six water molecules with it. At most, the ion need only contain two ions when transporting some cations such as cesium. However, for less permeable ions such as sodium, there is never more than one ion in the channel at a given time.

What is most interesting is that the more Dr. Anderson is able to observe the behavior of naturally occurring membrane channels the more they have resembled synthetic, well-studied channels like those from gramicidin A. There is much evidence that for some classes of channel in nerves and muscles, single-file transport is most likely. The study of the gramicidin channel is continuing to serve as a valuable model much as it has over the past 15 years.

"The Mobility of Erythrocyte Membranes"

R01 GM 28250-03, R01 GM 23585-06 (Koppel, D. E.), University of Connecticut, R01 GM 28832-03 (Peterson, S. W.), Boston University, R01 GM 27171-02 (Langdon, R. G.), University of Virginia

The technique of fluorescence redistribution after photobleaching (FRAP) is an established if not common technique for measuring the mobility of proteins in plasma membranes. As developed by NIGMS grantees Drs. Elliott Elson and Watt Webb, it involves attaching fluorescent label to membrane molecules either covalently or via specific antibodies. A small spot of the membrane is bleached by a laser flash and the diffusion of label into the bleached area followed. This yields data on the mobility or effective diffusion coefficient of membrane components. Some questions have been raised that the process of photobleaching itself changes mobilities by crosslinking membrane protein. Dr. Dennis Koppel has shown that for erythrocyte ghosts any such crosslinking does not alter the lateral mobility of proteins in the plane of the membrane.

Dr. Koppel has measured the mobility of integral membrane proteins such as the chloride channel, Band 3, in erythrocytes before and after physical and chemical removal of the membrane-associated cytoplasmic protein spectrin. Removal of spectrin increases the mobility of integral proteins fifty fold. Dr. Koppel has recently expanded these measurements to look at both protein and lipid mobility in the intact erythrocytes from normal and spectrin-deficient mice. These results strongly confirm the hypothesis that spectrin is an essential factor in limiting the mobility of both lipids and proteins in the membrane. In some yet-to-be-defined way the spectrin, or actin-spectrin matrix, hinders motion of the entire membrane.

Spectrin has generally been perceived as a structural protein whose effect on cellular physiology and biochemistry is likely to be indirect. Any effect on membrane or membrane protein behavior in view of the above findings would lead to study of the relationship between membrane fluidity and function. Recently Dr. Robert Langdon has shown that removal of spectrin results in the loss of a calcium-stimulated, magnesium-dependent membrane ATPase. This activity is specifically reconstituted by addition of purified spectrin to spectrin-depleted membranes. In a separate study, Dr. Scott Peterson has been studying down-regulation of insulin receptors after incubation of erythrocytes with insulin. In other words, in a comparatively very simple and well-defined system, the isolated erythrocyte, the effect of a hormone on the number of membrane receptors can be studied. In fact, this down-regulation can even be observed in erythrocyte ghost cells. It appears that spectrin depletion reduces or limits this down-regulation. Clearly, this matrix protein has profound effects on membrane function, and these and several other investigators are now pursuing the connection between membrane mobility and function.

"Structural Studies of Cellular Assemblies of Actin"

R01 GM 26357-04 (DeRosier, D.), Brandeis University

The characteristic most of us associate with life is movement. At the cellular level, there is a wide variety of motions, including swimming, crawling, and changes in cell shape. There is still no clear picture of either the mechanisms or the different roles played by the contractile proteins in producing movement. What understanding we have of the roles and mechanisms of actin, tubulin, and their ancillary proteins, comes largely from the study of specialized cells. For example, the sperm's flagellum has provided insights into the way in which tubulin, together with dynein, can generate movement. Both tubulin and actin may have roles in mitosis, the division of a cell into two daughter cells. Likewise, the muscle cell has provided information that has explained how actin, in conjunction with myosin, produces motion. The striated muscle model, with its unique, highly ordered array of proteins, is not able to account for all aspects of movement. New model systems are needed to elucidate the other mechanisms of actin-based motility.

One actin action system has been studied by Dr. David DeRosier, in close collaboration with Dr. Lewis Tilney of the University of Pennsylvania. This is the extension of the acrosomal process in the sperm of Limulus, the horseshoe crab. On contact with the egg, the sperm explosively generates a 60 micron long extension which carries the cytoplasmic membrane of the sperm to the membrane of the egg by penetrating the viscous jelly surrounding the egg. In the unfired sperm, the bundle lies coiled at the base of the sperm. The extension is effected by an uncoiling of the bundle, which is composed of about 80 actin filaments joined together by two other proteins. This motion is quite different from that of the muscle, where there is a telescoping action of one set of filaments relative to the others.

Over the past several years, Dr. DeRosier has examined this bundle in detail, using optical diffraction analysis of electron micrographs. It is an excellent structure for study since it is highly ordered and, therefore, amenable to the techniques of image analysis. During his postdoctoral work in the late 1960's in Cambridge, England, Dr. DeRosier with Dr. Aaron Klug developed the principles and original systems for three-dimensional reconstructions from electron micrographs. This work has led to the important field of image processing, and Dr. DeRosier remains one of its most creative and productive proponents. His application of these techniques to the Limulus sperm bundle has led to an understanding of the organization of filaments in the bundle and the mechanism of its extension.

In the bundle, the actin filaments are arranged hexagonally with their subunits in perfect transverse register. This organization has subsequently been shown to apply to bundles of actin from other structures such as the filopodium. An unusual feature of the organization in the coil is that the bundle is not smoothly bent but is polygonal, consisting of 14 straight sides and 14 bends. He has shown that: the bend is actually built into the bundle by a rearrangement of the interfilament bonds; and the 154 degree angle of the bend is specified by a combination of the helical symmetry of the actin filament and the hexagonal geometry of the bundle.

Structural analysis of the three states of the bundle--coil, false discharge, and true discharge--has determined details of these structures and led to an explanation of the mechanism of the extension. Dr. DeRosier has found a counter clockwise twist of the actin helix of .23 degree per subunit in the coil relative to that in the true discharge. This twist in the filaments, which is probably initiated by the binding of a small molecule, drives the extension. In both forms the bundle is a rigid cross-linked structure and thus the force in the bundle is greatly multiplied. Through computer based modeling, Dr. DeRosier has shown that the twist leads to a change in supercoiling which causes the bundle bends to rotate by 90 degrees, generating the extended form of the bundle. In the final step, which occurs concomitantly with extension, the bonding in the bends is rearranged, resulting in a perfectly straight bundle. This makes the extension irreversible.

This structure reveals an entirely new mode of actin-based motility which is completely different from that observed in muscle. In muscle, the actin filaments are moved by the ratcheting action of the myosin crossbridges. In the bundle, however, extension is driven by a change in the twist of the actin filaments. Such a change in twist arises from a change in bonding between subunits within the same filament. The bonding between filaments, on the other hand, serves to couple together the forces generated by the individual filaments. Dr. DeRosier believes that similar changes in twist are found in other kinds of bundles. He is now examining the microvillus, an actin containing extension found in a variety of cell types. He will apply the ideas gained from his studies of the Limulus sperm to these structures.

"Control and Guidance of Tissue Cell Locomotion"
R01 GM 24251-06 (Harris, A.), University of North Carolina, Chapel Hill

The unique aspect of the research being done in Dr. Albert Harris' laboratory is the culturing and observation of cells on special artificial substrata prepared in his laboratory. These substrata are designed to reveal various aspects of cell propulsion and guidance. The thrust of this work has been to find mechanical explanations for such events as gastrulation, nerve outgrowth, abnormalities of cancerous cells, and the like. In earlier studies, Dr. Harris used vacuum evaporation methods to make adhesion gradients and other patterns of controlled adhesiveness, and substrata (often glass) of different curvatures, groove sizes and other properties. The most recent technique developed in his laboratory utilizes a special silicone rubber sheet which is thin enough to be distorted and visibly wrinkled by the traction forces exerted by individual tissue culture cells. The basic method is to create a thin silicone rubber substratum by flaming silicone fluid. Sheets as thin as a micron are prepared on glass coverslips. The advantages of these sheets are their inertness to biochemical changes--especially shrinkage--non-toxicity, and transparency. The absolute magnitude of the forces exerted by the various cells is estimated by using glass microneedles whose flexibility is calibrated by hanging small paperweights on their ends. A micromanipulator is used to push the tips of these needles laterally upon the rubber until this surface is distorted to the same degree as it is by the cells. The shear force required is determined by observing the degree of lateral bending of the microneedle and comparing this with the bending produced by the weights.

Utilizing these measurements in conjunction with polarized light optics, interference reflection microscopy, and time lapse films, the silicone rubber substrata showed that cultured cells propel themselves by exerting shearing forces which are transmitted through the plasma membrane, tangential to its surface in the areas where it adheres to the culture substratum. This tangential shear force (= traction) was shown to vary widely among differentiated cell types; e.g., fibroblasts were 1000 times stronger than macrophages or granulocytes. In fact, observations of a spreading fibroblast show that its traction is sufficient to propel a cell 100-1000 times faster than needed for normal cell locomotion. The pattern established in these studies indicated that the more invasive cell types exert traction which is weaker than less invasive cells. Strength of traction was found to be inversely related to invasiveness. This pattern was evident not only among the differentiated cell types studied, but also between transformed and non-transformed cells; i.e., transformed cells exert weaker traction than non-transformed cells. This can be correlated with other properties of transformed cells, such as the ability to grow to high cell density in monolayer and to grow in suspension. Further support for the relationship of traction to other properties was provided when Dr. Harris' laboratory showed that reversal of transformation of CHO cells by treatment with cyclic AMP and testosterone strengthens their traction. Dr. Harris has also observed that contractile forces can rearrange fibers into large scale patterns resembling tendons or organ capsules, and that explantation leads to a strong increase in contractile force, perhaps related to the phenomenon of wound contraction.

Research on non-muscle cells implicates cytoplasmic actins, myosins and other related proteins in cell movement. Despite the accumulation of data regarding the molecular properties of these proteins and their spatial distributions within fibroblasts and other tissue cells, it is still not known how the contractile forces of these proteins are transmitted through the plasma membrane to produce cell locomotion and other effects. Dr. Harris' laboratory has now designed experiments to gain insight into these problems and is currently examining how the forces of cytoplasmic contraction are transmitted through the plasma membrane to adherent objects on the outside.

This work involves two main areas of medical significance: tissue contraction around deep wounds and implanted prostheses, and the problem of cellular invasiveness, specifically in relation to cancer.

Regarding the first area, data from this laboratory support the interpretation that tissue contraction results from an increase in fibroblast contractility rather than a contraction of intercellular collagen as has been proposed by other workers. The data suggest, however, that the force is exerted as a shear along the cell surface applied to collagen, rather than by the contraction of the cells themselves. The use of flexible substrata to monitor changes in cell contractibility could eventually provide a useful tissue culture system for testing treatments designed to reduce burn contracture, or alternately, to promote wound contractions. Studies on cellular invasiveness will be extended to determine what normally prevents solid-tissue cells such as fibroblasts from moving as invasively as leukocytes or what physical changes in cancerous cells tend to render them invasive. Recent data by Dr. Harris, and by Dr. Steinberg, implicate changes in contractile strength. Dr. Harris will utilize chicken fibroblast cells transformed by a temperature sensitive Rous sarcoma virus for extension of earlier studies which compare cell contractility of non-transformed and transformed cells.

"Membrane Proteins and Development"
R01 GM 26968-04 (Noll, H.), Northwestern University

The orderly integration of cells into tissues during embryogenesis is central to the production of complex organisms from the fusion of egg with sperm. Recent findings suggest that tissue specific cell adhesion in development is mediated by specific recognition proteins in the membrane. The interest in molecules responsible for cell adhesion is also stimulated by the hope that their study would lead to an understanding of the nature of positional information and its consequences for differentiated function. The recent discovery by Dr. Marshall Nirenberg, building on the work of others such as Glaser (GM

18405), McClay (GM 22477) and Lilien (GM 29131), showing that membrane proteins form gradients reflecting morphological organization, is the first evidence in support of this concept. Dr. Hans Noll's work on two different Italian sea urchin species has pin-pointed a role for specific membrane proteins in aggregation, and demonstrated a possible controlling link to DNA synthesis.

Upon fertilization, sea urchin eggs develop in perfect synchrony through a series of morphologically distinct stages (blastula, gastrula) to the final larval form called the pluteus. During this period, in which complicated structures consisting of skeleton, digestive tract, and organs of locomotion are formed, total cell mass does not increase although the cell number increases from one to several thousand.

Blastula embryos of the sea urchin Paracentrotus lividus can be dissociated into single cells by exposure to Ca^{+2-} and Mg^{+2-} free sea water. The dissociated cells can, when removed to normal sea water, reassociate spontaneously to form aggregates capable of development to the pluteus. Aggregation is prevented by Fab fragments obtained by immunization with purified membranes from blastula embryos. The Fab inhibition of aggregation is reversed by soluble proteins extracted with 2.5 percent butanol from either purified membranes or from intact cells. These butanol extracts also strongly stimulated the rate of reaggregation of cells dissociated in the absence of Fab fragments.

Exposure of dissociated cells to butanol completely removed the protein(s) responsible for reaggregation of the cells without impairing their viability. Reaggregation and embryonic development were completely restored to the extracted cells by readdition of the butanol extracted proteins from either membranes or cells. Thus, Dr. Noll has demonstrated that proteins essential for aggregation can be removed from membranes of living cells and then reinserted with the full restoration of cellular function.

It had previously been demonstrated by other workers that dissociated blastula cells of Paracentrotus were unable to synthesize DNA if prevented from aggregating by dilution in sea water. DNA synthesis is restored when cells are allowed to reaggregate. Dr. Noll demonstrated that butanol extraction, which causes the absence of intercellular contact, not unexpectedly produces a block of DNA synthesis. However, addition of Fab fragments against purified membranes, at a concentration which completely prevents reaggregation, restores DNA synthesis. Thus, as far as DNA synthesis is concerned, the binding of Fab fragements to the cell membranes acts like intercellular contact. This is consistent with a mechanism of DNA synthesis regulation based on allosteric modulation of a membrane protein(s). Dr. Noll is currently purifying the proteins necessary for aggregation, and will use the Fab fragments to purify the proteins which, when stimulated by binding, allow DNA synthesis to occur. He is also establishing the generality of this finding in other cell types.

"β-Lactamase and its Inhibitors"
R01 GM 21659-09 (Knowles, J.), Harvard University, R01 GM 21514-08
(Gilbert, W.), Harvard University

β-Lactam antibiotics exert their lethal effects on growing cells by interfering with the enzymes responsible for the synthesis of the bacterial cell wall. Resistance to the bacteriocidal action of β-lactams may develop, however, and this resistance is most frequently due to the production by the infecting organism of a β-lactamase which catalyzes the hydrolytic cleavage of the β-lactam ring in a penicillin or cephalosporin. The gene that encodes the β-lactamase is often carried on a plasmid, and the promiscuous transfer of plasmids among becteria has sharply increased the incidence of β-lactam-resistant infections. This problem is of obvious clinical importance and has been attacked primarily through the development of β-lactam antibiotics that are less sensitive to the hydrolytic action of the β-lactamases. More recently, a second approach to the β-lactamase problem has been taken; this approach utilizes the synergistic action of compounds that inhibit the β-lactamase when administered simultaneously with a good (though β-lactamase-sensitive) antibiotic. The first β-lactamase inhibitor, clavulanic acid, in combination with Amoxicillin, is currently in a clinical trial. In 1976, the discovery of clavulanic acid was followed by the isolation or synthesis of a number of other β-lactam derivatives which are effective β-lactamase inhibitors. Studies supported by NIGMS have produced valuable information concerning both the reaction pathways of these inhibitors and the amino acid sequence and active-site structure of the β-lactamase.

One of the central figures in these studies is Dr. Jeremy Knowles (GM 21659) who has obtained extensive evidence on the pathways by which these inhibitors interact with the β-lactamase. From his work on the penam sulfones, Dr. Knowles has concluded that there are three types of interactions of these inhibitors with the enzyme. He has been able to gather enough kinetic, spectroscopic and chemical data to support these schemes.

The amino acid sequence of the β-lactamase which catalyzes these reactions has also been determined. Dr. Knowles suggested to Dr. Walter Gilbert and an associate of his, Dr. Gregor Sutcliffe, that they determine the DNA sequence of the plasmid-borne gene for the enzyme. Dr. Sutcliffe, working with NIGMS support through Dr. Gilbert's grant (GM 21514), was able to isolate the gene and experimentally determine the base sequence of both its coding and complementary strands. (The entire project required less than eight months!) From a consideration of amino acid sequence information, it is now clear that the β-lactamase gene codes for a protein of 286 amino acid residues; however, the first 23 residues do not appear in the mature enzyme and are presumed to form a signal for secretion from the cell. The sequence data will facilitate work being conducted by Dr. J. R. Knox to determine the X-ray crystallographic structure of the β-lactamase.

A number of events and issues of the last few years add significance to these and other studies of β-lactamase and its inhibitors. For example, the plasmid that encodes β-lactamase has been present in the pathogenic Hemophilus influenzae b strain since 1974. With this change, diseases due to H. influenzae (e.g. pneumonia, otitis media, meningitis) have become more serious and wide-spread. The plasmid has also been found in the venereal disease producing strain of Neisseria gonorrhoeae. This and the finding that the syphilis-producing Treponema pallidum has the capacity to acquire plasmids have raised the specter that the principal drug (penicillin) used in the treatment of the common venereal diseases may lose its efficacy. Finally, the somewhat indiscriminate and loosely-controlled use of the β-lactam antibodies for humans in many Third World countries as well as the large-scale addition of these antibiotics to livestock feed in this country create selection pressures that favor the growth of populations of β-lactam resistant organisms. Given these circumstances, vigorous research on β-lactamase and its inhibitors seems particularly well-justified.

"AMP Nucleosidases"

R01 GM 21083-07 (Schramm, V.), Temple University

The adenine nucleotides AMP, ADP, and ATP are widely used in metabolic processes to accept, store, and transfer chemical potential energy and to serve as integrators of many of these processes by modulating the activity levels of some of the participating enzymes. As might be expected, cells, in adopting this triad of compounds for these central roles, also evolved sensitive systems for the regulation of the total intracellular concentrations of these nucleotides as well as the ratios of their concentrations. The importance of these parameters is evident from the fact that numerous individual enzymes and certain entire pathways respond to changes in ATP or AMP concentration or to the ratio of these concentrations. These responses are consistently in the right direction so that optimum levels of ATP can be maintained. For example, regulatory enzymes of the ATP-regenerating sequences (phosphofructokinase, pyruvate kinase, pyruvate dehydrogenase, citrate synthase, and isocitrate dehydrogenase) are depressed in activity as the ATP/AMP ratio rises, while other enzymes consuming ATP (aspartokinase, citrate cleavage enzyme, phosphoribosylpyrophosphate synthase) are activated as this ratio increases. Thus, it is clear that all factors which contribute to the regulation of intracellular adenine nucleotides concentration and ratios have an important impact on many cellular systems.

One such factor for prokaryotes is AMP nucleosidase--an enzyme that catalyzes the cleavage of AMP to ribose-5-phosphate and adenine. While investigations of the functions of AMP nucleosidase in prokaryotic metabolism are still in progress, it is now evident that this enzyme plays the major role in AMP degradation. Thus, with the exception of the loss of adenine nucleotides to biosynthetic pathways (RNA & DNA primarily), AMP degradation by the nucleosidase is the mode by which prokaryotes balance the *de novo* production of adenine nucleotides; it is in this fashion, then, that AMP nucleosidase participates in the regulation of intracellular concentration of adenine nucleotides.

Present knowledge of AMP nucleosidase biochemistry, including its metabolic role, physical and chemical properties, has been developed largely by Dr. Vern Schramm of Temple University with support (GM 21083) from the NIGMS. The enzyme was originally identified by Hurwitz, Heppel and Horecker, but it has been intensely studied by Dr. Schramm and his co-workers for some 10 years. They have found that the enzyme consists of six apparently identical subunits which dissociate to three inactive dimers in the presence of phosphate. Electron microscopy of the active enzyme gives an appearance similar to a three-leaved clover 108 Å in diameter with a central hole. From these structural studies, as well as kinetic experiments, it was determined that there is a catalytic and regulatory site for each dimer in the functional hexameric enzyme. The compounds which have been identified as regulators of this allosteric enzyme are MgATP (activation) and phosphate (inactivation). Dr. Schramm has carried out magnetic resonance studies with analogs of both the substrate and the regulators; these studies have revealed that the catalytic site and allosteric site are separated by more than 20 Å. The catalytic mechanism of AMP nucleosidase appears to involve a dissociative, or S_N1, process. The mechanism, consistent with stereochemical and secondary isotope effect data, involves close interaction of the enzyme with both the adenine and ribose-5-

phosphate portion of AMP followed by a conformational change which wrenches the two portions of the substrate molecule apart. The reactive ribose carbon which is generated is then attacked by an enzyme-bound water molecule.

Dr. Schramm has observed that most prokaryotes contain AMP nucleosidase, while eukaryotic organisms, including yeast, fungi, algae, and mammalian tissue do not. However, mammalian tissues, yeast and fungi are known to contain AMP deaminase, an enzyme which also degrades AMP (AMP + $H_2O \longrightarrow$ IMP + NH_3). Since analysis of prokaryotes for AMP deaminase has yet to reveal any that contain this enzyme, Dr. Schramm has proposed that the division between prokaryotes and eukaryotes is also the division for regulation of AMP levels by the AMP nucleosidase or AMP deaminase. Characterization of yeast AMP deaminase in Dr. Schramm's laboratory showed that it is regulated similarly to AMP nucleosidase; i.e., it is activated by MgATP and inhibited by phosphate. Thus the metabolic role and the regulatory controls of these enzymes are directly related. Although these relationships certainly do not necessitate that the deaminase gene is an evolutionary descendant of the nucleosidase gene, Dr. Schramm is pursuing sequencing studies of the two structural genes in an effort to clarify this point.

These studies, which began simply as a study of an enzyme mechanism, are leading to a more complete understanding of the ways energy metabolism in both prokaryotes and eukaryotes is regulated.

Cellular and Molecular Basis of Disease Program

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GENETICS PROGRAM

OBJECTIVES

The Genetics Program, one of the components of the National Institute of General Medical Sciences (NIGMS), is charged with the support of a broad range of research activities on the fundamental mechanisms of inheritance. The Program is committed to understanding basic concepts of genetics at the chemical, cellular, organismal, and population levels. Such information forms the foundation on which clinically relevant efforts at prevention and treatment of human genetic diseases are based. This program achieves its major objective through the support of research and research training in basic genetic problems. The Genetics Program does not generally support work directed at specific disease entities, but the following examples reveal the extent to which recent research supported by the program has been translated into knowledge and methodology of clinical significance: 1) The conceptual and technical development of electrophoretic techniques and procedures using restriction enzymes has extended the ability of geneticists to diagnose defects at the single gene level. Scientists, for example, can now use these methods to detect the presence of the abnormal sickle cell allele in individuals at risk for the disease. 2) The highly sophisticated technology of cloning and expressing single genes from the human genome in microorganisms has made it possible to prepare critically needed therapeutic reagents, including growth hormone, somatostatin, insulin, and interferon. These reagents can now be synthesized from human genes by genetic technology in large quantities and at reasonable prices.

Another objective of the Genetics Program is to integrate genetic approaches with those of other disciplines. One way that it achieves this aim is by stimulating interactions among the various Institute programs. For example, the bioenergetics portion of the Cellular and Molecular Basis of Disease Program and the mitochondrial genetics portion of the Genetics Program are administered by the same person. An integrated view of the application of genetics to the study of mitochondrial function is therefore possible. This area is, in fact, proving to be exciting and active, and scientists are making considerable progress in dissecting and understanding mitochondrial function.

Similarly, the Pharmacology and Genetics Programs have been aware of the evolving and reviving interest in the relationship between pharmacology and genetics. As a result, considerable interprogram dialogue has been stimulated, and the two programs collaborated on a workshop this year to consider issues relating to the newly emerging field of molecular pharmacogenetics. The Genetics Program hopes to continue such collaborative and interactive arrangements in the expectation that they will facilitate an understanding of the importance of genetics to other disciplines.

ORGANIZATION AND STAFFING:

The Genetics Program of the NIGMS is organized into two sections that are designed both to lend emphasis to the current directions in the broad field of genetics and to take advantage of the strengths of the program staff. The Molecular and Medical Genetics Section supports research in the areas of the physical and chemical nature of nucleic acids; the mechanisms of replication, transcription, and translation; behavioral genetics; and cytogenetics and gene mapping. The section is headed by Dr. David Beck, and the professional staff includes Drs. George Woolley, Barbara Williams, Irene Eckstrand, and James Cassatt. Dr. Eckstrand, an expert in the areas of ecological genetics, behavioral genetics, and the use of Drosophila as an experimental organism, had been a part of the Genetics Program as a visiting exchange scientist under the Intergovernmental Personnel Act. In June 1982, Dr. Eckstrand became a permanent employee of the NIGMS. A physical chemist by training, Dr. James Cassatt served as Executive Secretary of the Molecular and Cellular Biophysics Study Section in the Division of Research Grants for three years. In May 1982, Dr. Cassatt joined the Genetics Program.

The second section of the program is the Genetics of Growth and Differentiation Section. This section was formed to focus on areas as seemingly diverse as cellular differentiation, morphological development, chromosome mechanics, and the rearrangement of genetic elements. However, the common thread that links these diverse areas is the temporal and spatial control of gene expression. Dr. Carl Rhodes, who previously worked part-time in the section while serving as an Executive Secretary in the NIGMS Office of Review Activities, joined the Genetics Program full-time in June 1982, and is now serving as Acting Chief of this section. The Section's professional staff includes Drs. Dorothea Miller, Fred Bergmann, and Judith Greenberg. Dr. Bergmann, in addition to his duties as Program Director, administers a small portion of this section's portfolio. In December 1981, substantial strength was brought to the section by the addition of Dr. Judith Greenberg. Dr. Greenberg is a developmental biologist by training, and she came to the NIGMS from the intramural program of the National Institute of Dental Research where she did research on the differentiation and migration of neural crest cells.

The addition in the past year of four excellent full-time members, Drs. Cassatt, Eckstrand, Greenberg, and Rhodes, brings the Genetics Program back to full strength, replacing losses of staff in the previous year. In January 1982, Dr. Elke Jordan, a member of the Genetics Program for six years and its Deputy Director for four years, was named Associate Director for Program Activities for NIGMS. Dr. David Beck, a member of the program since 1978, was appointed its Deputy Director in January 1982.

CONTRACT

The Human Genetic Mutant Cell Repository, Contract NO1-GM-9-2101, Institute for Medical Research, Camden, New Jersey.

The Human Genetic Mutant Cell Repository, known as the Cell Bank, was established for the dual purposes of stimulating and facilitating research on human genetic disorders. This facility stimulates human genetic research by providing investigators with cell lines from patients with common or rare hereditary diseases. Thus, scientists are able to study disorders by using cells with which they might not ordinarily work or to which they would not have access in their own research settings. If such cells were not available "off the shelf" from the Cell Bank, opportunities for exploring new approaches to human genetic diseases might be lost. The Cell Bank facilitates research by providing cell lines which are well-characterized, are free of contamination with mycoplasma or with other cell lines, and are at an early stage of their useful growth potential, before the onset of possible chromosomal changes.

Analysis of the use of the Cell Bank indicates that cell lines are being distributed widely. Thus, it appears that the Bank's major goal is being met. The most recent data show that 613 lines were received or expanded and 3972 cultures were distributed in 1981. To determine more accurately the success of the Cell Bank in achieving its goals, NIGMS has begun an evaluation of the Cell Bank's performance with respect to the investigators who use the cells and the quality of the results they obtain. The study is being conducted by both bibliometric techniques and evaluation by an advisory committee.

In order to be most responsive to the needs of the scientific community, the Cell Bank must update its collection of cell lines as new areas of research emerge. Under the guidance of a distinguished advisory group, chaired this year by Dr. C. Thomas Caskey, the Cell Bank has considered or initiated several special collections. In the past year, the Advisory Group has given high priority to a collection of lines from patients with genetic ophthalmological disorders.

The Cell Bank has continued its efforts to expand its cell lines relating to neurodegenerative disorders, connective tissue diseases, and X-chromosome abnormalities. Major acquisitions, such as the affective disorders collection and the Huntington's disease collection, have been completed. In particular, the Huntington's collection contains a number of lines from a highly inbred population in Venezuela. This raises the exciting possibility that, if individuals homozygous for the dominant Huntington's gene are viable, cells carrying a double dose of this gene may now be among the cell lines in the collection.

Collections such as those for Huntington's disease and the affective disorders raise special problems in cell banking. To maximize their potential usefulness, such collections must include cells from extended pedigrees. However, since the current demand for the complete set of these lines might be very low, the cost of supplies and staff to expand all the samples immediately is wasteful. For this reason, the Cell Bank uses a procedure, dubbed "freeze and hold," whereby a skin biopsy is placed directly into storage in liquid nitrogen. Experimentation by the Cell Bank has shown that cells from biopsies treated in this way are fully viable when thawed. Thus, tissues may be stored until a need for them arises, and costs are thereby reduced. Unfortunately, due to the limited viability of B lymphocytes, a similar procedure is not possible for the corresponding blood samples. For the lymphoblast cultures, therefore, limited expansion is allowed when demand for a line is expected to be low.

RESEARCH HIGHLIGHTS

This year's report of research highlights is divided into two sections -- "The Return of *Drosophila* as a Genetics Research Tool" and "New Insights into Gene Organization." Although other important advances which do not fit into these categories have been made, these two areas of research have been especially exciting in recent years and have emerged as common threads that unify the diverse subsections within the program. This format emphasizes important features of the program. First, it shows that the program's subsections are interactive and share similar interests and research activities. Collaborations among staff members and among investigators in these areas have been, and continue to be, of great value. Second, it demonstrates that the importance of a basic research project may not be obvious in its early stages. Most research featured in this report is built on years of careful work, the importance of which was not always apparent when the work was begun. Similarly, the directions of future work and the applications of current basic research efforts cannot be predicted easily now. Thus, this research is built upon earlier work and, in turn, will form a solid foundation for future research.

1. THE RETURN OF DROSOPHILA AS A GENETIC RESEARCH TOOL

Introduction

When Thomas Hunt Morgan began his famous studies on the genetics of the common vinegar fly, *Drosophila melanogaster*, he surely had little idea of how valuable those small creatures would become. Today, well over 1000 species of *Drosophila* have been identified (over 300 unique species in the Hawaiian Islands alone) and characterized down to the last bristle and wing vein. We probably know more about the genetics, morphology, physiology, ecology, and biochemistry of *Drosophila* than of any other organism, with the possible exception of the bacterium, *Escherichia coli*.

T.H. Morgan was not the first to undertake genetic studies of the vinegar fly, although he is often given credit for doing so. In fact, William Castle of Harvard University had been studying inbreeding in *Drosophila* for a number of years, but he noticed no mutations. Castle recommended the flies to his entomologist friend, Frank Lutz, who identified at least one mutation before recommending the flies to Morgan. Two years later, Morgan was throwing up his hands in despair over the possibility of obtaining any useful results from the fly work. Then, in May, 1910, the most famous insect in science -- the white-eyed *Drosophila* male -- appeared. Morgan recognized the fly's potential and carried the white-eyed male and a few normal females home in a bottle every night until they produced offspring.

From the subsequent experiments conducted by Morgan, Bridges, Sturtevant, and Muller in the famous Fly Room at Columbia University, the skeleton of modern genetics was constructed. Drosophila proved to be a useful experimental organism for many reasons. Its generation time is short (D. melanogaster's life cycle is 10-14 days), and flies are easily maintained in large numbers in the laboratory.

In the 1920's Muller showed that X-rays could cause mutations, and researchers began to develop lines of Drosophila with mutagen-induced genetic variations. The mutant lines now available range from chromosome deletions and structural rearrangements to nucleic acid substitutions that lead to minor changes in the structures of enzymes. Well over 4000 different mutant lines are now available upon request to researchers. By comparing the normal with the mutant forms, scientists can often deduce the roles and importance of specific genes and enzymes.

Although D. melanogaster is the best characterized Drosophilid, many other species have also played important roles in the development of genetics. Drosophila species are ubiquitous, occurring in nearly every locale except New Zealand. Their habitats range from the insides of hermit crab shells to the Arctic tundra. Because they are not agricultural pests, Drosophila species can be easily transported across state and national borders. Thus, the vinegar fly is ideal for studies on population structure and evolution. Between the thousands of known mutants of Drosophila melanogaster and the variety of available Drosophila species, there is a Drosophilid to suit nearly any research purpose in genetics.

Prior to the Second World War, Drosophila was the principal research tool of geneticists. It has been said that if one wants to study rabbit genetics, look at rabbits; if one wants to study genetics, look at Drosophila; if one wants to study human genetics, forget it. Unfortunately, this view prevailed for many years and probably slowed work on other systems. However, some geneticists and microbiologists joined forces in the 1930's and began extraordinarily successful studies of microbial and viral genetics. Because these scientists used simple prokaryotic organisms, and because they were extremely creative and careful in their research plans, genetics has made great strides over recent years. Our understanding of DNA structure, transcription, control of gene expression, and chromosome replication comes, in large part, from these studies. In fact, for some years, microbial genetics has somewhat overshadowed studies on Drosophila. Because of their simple structure, bacteria and viruses have been particularly useful in the elucidation of basic genetic processes.

The work on prokaryotic systems has produced a sound foundation of both information and techniques which can now be applied to eukaryotic systems. Because of the amount of information and the vast array of mutants and naturally-occurring species, Drosophila is again emerging as an important research tool. These flies are now being used as animal models to test genetic hypotheses on subjects ranging from biochemistry to evolution. The following grantee accomplishments highlight ways in which Drosophila is now being used in genetics research.

DNA Replication

"Recent Studies of Prokaryotic and Eukaryotic DNA Replication Systems Reveal Important Differences"

R01 GM 07581-21 (Kornberg, A.), R01 GM 06196-23 (Lehman, I.), Stanford U.

Extensive studies on the enzymology and mechanics of DNA replication in prokaryotes have been very productive in elucidating chemical pathways involved in DNA replication. Initiation and regulation of DNA replication are of great interest because these processes control cell growth and differentiation. Thus, understanding how cells replicate their DNA could shed light on such problems as carcinogenesis, mutation and repair, and embryonic development. Unfortunately, our understanding of these processes has been slow to develop, due mainly to the complexity and intricacy of the replication system which must ensure exact duplication of every DNA molecule during each cell cycle.

This year, Dr. Arthur Kornberg succeeded in developing a cell-free system which can initiate DNA replication. Using recombinant DNA technology, Dr. Kornberg and his colleagues created a plasmid of 12,000 nucleotide bases, named pSY317, which contained the origin of replication (oriC) from an Escherichia coli chromosome. This 245 base pair oriC region made the plasmid functionally very similar to, but structurally much smaller than, a normal E. coli chromosome. This, in effect, rigged the pSY317 system in favor of initiation of replication rather than elongation of the DNA chain.

Dr. Kornberg and his colleagues had to tamper creatively with the chemical matrix before they hit on the right combination of enzymes, salts, and temperature to support replication. Subsequent studies have confirmed that the enzymes in the Kornberg system behave much as they would in normal cells, and that the pSY317 plasmid replicates much like a normal chromosome.

Many years of creative and detailed work by hundreds of scientists preceded Dr. Kornberg's success. Studies on the structure and kinetics of replication enzymes, on the mechanics of DNA winding and unwinding, on cell growth and development, and on plasmid organization and construction contributed to this work. The results of these studies are providing a foundation for studies of DNA replication in eukaryotic cells.

Eukaryotic cells are much more complex than are prokaryotic cells. In eukaryotes, the chromosomes are long strands of DNA which are carefully packaged with special proteins to fit into the nucleus of a cell. Prokaryotic cells have no nucleus, and the single chromosome in each cell is usually circular. Each prokaryotic chromosome has a single origin of replication; each eukaryotic chromosome has many origins. Recent studies suggest that the enzymes which direct replication may be very different in the two groups of organisms.

Dr. I.R. Lehman, a leader in identifying and characterizing replication enzymes in E. coli, has lately turned his expertise toward understanding the more complex machinery of eukaryotes.

Dr. Lehman chose D. melanogaster as his test organism because at certain developmental stages the larvae show a very rapid rate of replication. In fact, the entire genome replicates every three to four minutes in the early embryo. This suggests that replication complexes are abundant and that large amounts of enzyme could be isolated and purified. Further, during these active stages, the chromosomes have an unusually high density of replication forks, approximately one for every 8-10 kilobases. Thus, D. melanogaster larvae appear to be an ideal system for studying replication in eukaryotes.

Dr. Lehman's ultimate goal is to purify and characterize a group of enzymes that will replicate a D. melanogaster chromosome. However, because eukaryotic chromosomes are very complex, Dr. Lehman chose to study the actions of D. melanogaster enzymes on M13 bacteriophage DNA, which is a single-strand loop. This approach allows him to study the properties of eukaryotic enzymes without the additional complexities of eukaryotic chromosomes.

The D. melanogaster system first proved its value when the laboratory was able to purify DNA polymerase in milligram quantities rather than in the microgram quantities usually recovered from bacterial systems. The Drosophila enzyme was shown to consist of four subunits with approximate molecular weights of 148,000, 56,000, 46,000, and 43,000. The largest subunit is the catalytic core of the enzyme, associated with actual DNA strand elongation. The functions of the other subunits have not yet been clearly defined, but they appear to increase the ability of an enzyme to process DNA by staying tightly bound to the DNA strand.

Chemical purification of an enzyme sometimes results in proteolysis or partial destruction of the protein. This can lead to erroneous conclusions about the structure and characteristics of the enzyme. Anticipating this problem, Dr. Lehman has begun to use immunological techniques to obtain pure enzymes. He now uses antiserum against DNA polymerase to obtain intact, pure enzyme. The polymerase purified by this method has a large subunit with an approximate molecular weight of 200,000; the other subunits of the enzyme are also larger when purified by the immunological technique. This suggests that the original purification techniques destroyed part of the enzyme. Dr. Lehman is now developing the immunological technique in order to purify large amounts of enzyme and determine whether the intact enzyme has additional functions or activities.

Another replication enzyme is primase, which initiates DNA replication by synthesizing an RNA primer strand onto which the new DNA molecule is attached. Ultimately, the primer is removed from the DNA strand by other enzymes. Primase isolated and characterized in E. coli has a molecular weight of 60,000. Dr. Lehman has tried to purify the D. melanogaster primase -- so far without success. However, his failure to isolate a primase has led to a very exciting hypothesis. Primase apparently cannot be separated from polymerase regardless of the technique used to isolate the enzyme. It appears that the molecular complex containing polymerase also performs the primase function. Preliminary studies using yeast and rat liver suggest that this is true for other eukaryotes.

Dr. Lehman's studies on D. melanogaster have shown clear, but unexpected, differences between prokaryotic systems and eukaryotic systems. The finding that primase and polymerase functions are tightly bound to each other in the replication complex is already changing the traditional view of the replication process. Given the importance of DNA replication in embryonic development and aging, as well as in such diseases as cancer, these studies are especially important. A practical application of the studies is likely to be a new technique for purifying large amounts of replication enzymes. Further, the D. melanogaster larvae have proven their value as a source of material for these studies and as a model for eukaryotic DNA synthesis.

"A Fortuitous Collaboration Leads to Elucidation of The Molecular Basis of a Developmental Mutant"

R01 GM 28078-02 (Greenleaf, A.), Duke University, and R01 GM 13631-17 (Lefevre, G.), California State University, Northridge, California

The fields of enzymology and genetics may seem far removed from one another. There are occasions, however, when the enzymologist can use genetics to study enzyme mechanisms, especially through the use of mutants which have altered enzyme structure. For obvious reasons, most of these experiments have used microorganisms. Recently, however, Dr. Arno Greenleaf has made use of a Drosophila mutant to study the properties of one of the cell's most important enzymes, RNA polymerase.

Three distinct classes of nuclear RNA polymerase are involved in eukaryotic transcription. Polymerase I transcribes ribosomal RNA genes; polymerase II, which is sensitive to the mushroom toxin α-amanitin, transcribes structural genes; and polymerase III transcribes 5S and tRNA genes. With bacterial systems, two general approaches have been applied to study RNA polymerase subunit function -- mutants with altered RNA polymerase have been isolated and studied, and RNA polymerase has been reconstituted in vitro following dissociation of its subunits. Unfortunately, attempts at in vitro reconstitution of eukaryotic RNA polymerase have not been successful. However, genetic approaches have been applied successfully to the study of RNA polymerases in cultured cells and yeast. Now Dr. Greenleaf has been able to use a Drosophilia mutant to obtain an RNA polymerase II with altered function. This is the first such study in a multicellular organism.

The mutant, named C_4, was obtained following mutagenesis with ethylmethanesulfonate and selection for resistance to α-amanitin. The isolated mutant enzyme was found to be 250-fold less sensitive to inhibition by α-amanitin in vitro than was the wild type enzyme. The C_4 mutation mapped to a single genetic locus, and Dr. Greenleaf showed that several previously described recessive lethal mutations at this locus also altered the properties of RNA polymerase.

Dr. Greenleaf further investigated the structural changes which resulted in altered enzymatic properties. As noted above, α-amanitin was a much poorer inhibitor of C_4 polymerase activity than was the wild-type enzyme. A 200-fold difference in the binding affinities was consistent with this difference in inhibition. Other experiments demonstrated that the stabilities of the mutant and wild type enzymes were similar. Hence, the difference in resistance seems to be due to a structural change in the binding site of α-amanitin.

As a follow-up to these experiments, other mutants involving the same locus were produced. Unlike the original mutant, the new mutants were temperature sensitive. Further, unlike the original C_4 polymerase, enzyme from these new mutants was found to be less stable than was wild-type enzyme. Further research on these interesting mutants awaits purification of adequate amounts of the polymerase.

Perhaps the most interesting feature of this project relates to its implications for genetics. Dr. George Lefevre, Jr. has been studying mutation frequencies in Drosophilia for a number of years. Recently he discovered a new homeotic mutant, which he named ultrabithorax-like (ubl). Homeotic mutations alter the developmental pathways of certain organs. For example, the mutant, ophthalmoptera, changes the fate of presumptive wing cells to that of eye cells. Homeotic mutants have been characterized by a long-time NIGMS grantee, Dr. David Hogness (GM 20158-09). He and other researchers have shown that homeotic mutants are valuable research tools in studies of the genetics of development and differentiation. However, until now, the molecular basis of these mutations was unknown.

Dr. Lefevre mapped ubl and found it to be allelic with the RNA polymerase mutation described by Dr. Greenleaf. This fortuitous discovery suggests that ubl, and perhaps other homeotic mutants, may fail to undergo normal transcription as a result of an abnormal RNA polymerase.

This line of work may prove to be critical in understanding the molecular basis of development. It came about because of collaboration between two scientists, one an enzymologist and one a classical geneticist. The importance of such interactions is that results from different fields may be related in important and intriguing ways.

"Characterization of Transfer RNA in the King of Beasts"
R01 GM 22854-06 (Soll, D.), Yale University

Dr. Dieter Soll and his co-workers have used Drosophila to develop an understanding of the structure and expression of the genes coding for the family of transfer RNA (tRNA) molecules. Scientists have sequenced over 40 of these small (73-93 nucleotides) molecules and have established the 3-dimensional structures of many tRNA's. Each tRNA species is transcribed directly from a DNA template, and the many kinds of tRNA therefore correspond to a family of tRNA genes on the chromosomes.

Transfer RNA is essential to basic cellular processes. In protein biosynthesis, specific tRNA molecules bind to individual amino acids and transfer them to the ribosomes where proteins are assembled. However, tRNA also has many less well-known functions, ranging from control of gene expression at the DNA level to regulation of the gene for primary reverse transcriptase, the enzyme which transcribes DNA from an RNA template. In short, tRNA molecules have complex and diverse functions.

Despite the importance of tRNA's, their synthesis is not well understood. For example, it is commonly believed that tRNA levels reflect the amino acid composition of the proteins synthesized by the cell. Recent evidence now indicates that relative amounts of different tRNA molecules are regulated by the cell's use of messenger RNA rather than by its use of specific amino acids. Thus, some interesting and important questions about tRNA's are yet to be answered. In addition to the obvious importance of understanding tRNA because of its cellular roles, the tRNA gene system may become a useful model for studying control of transcription in general. The tRNA genes and their products are relatively small and can be studied and characterized more easily than can some larger gene systems.

Dr. Soll has recently shown that eukaryotic cells contain many copies of each tRNA gene. Present estimates of 7 repeats in yeast, 10 in Drosophila, 100 in the mouse, and 200 in the frog may, in fact, be far too low. Labeled tRNA molecules, specific for a single amino acid, hybridize to many regions of Drosophila polytene chromosomes, showing that many gene copies are located throughout the genome. The genes are often clustered, and different varieties may be intermingled. In Drosophila, some copies appear in tandem. Dr. Soll has shown that, even in these clusters, individual tRNA genes are independent transcriptional units.

Dr. Soll has also sequenced several tRNA genes. Early publications reported that some yeast tRNA genes contained intervening sequences, but Dr. Soll has shown that not all yeast tRNA genes have these sequences. As one might guess, tRNA coding sequences are often different in unicellular and multicellular organisms.

RNA polymerase III is responsible for tRNA synthesis. Dr. Soll has shown, however, that this polymerase is, by itself, unable to transcribe the correct tRNA genes from a purified DNA template. Dr. Soll's laboratory is now searching for additional protein factors which are necessary for tRNA synthesis. The primary control region for tRNA transcription by RNA polymerase III is within the coding region for mature tRNA. Dr. Soll has recently discovered an additional control region outside the known coding region. The relationship between the two control regions is not yet clear; however, the new region appears to regulate the activity of the internal region. After transcription, tRNA matures in a multistep process which involves excising intervening RNA sequences and splicing the coding regions. Although splicing has been studied for several years, the splicing enzyme has not been purified. The major difficulty has been that the original, unspliced transcripts are hard to isolate because they are both rare and short-lived. Dr. Soll has now developed an in vitro system for cloning tRNA genes to provide large amounts of mature tRNA precursor molecules. These tRNA's are transcribed and processed precisely as cellular tRNA's are.

Dr. Soll, using Drosophila as a model system, is answering some important questions about tRNA gene structure and expression. His results suggest that an understanding of eukaryotic gene expression and regulation will come, in part, from carefully and creatively building a solid foundation of basic information about tRNA genes.

"An Enlightened Hypothesis on the Structure of Polytene Chromosomes"

R01 GM 19179-11 (Laird, C.), University of Washington, Seattle

One of the most important tools for studying chromosome organization and structure has been the giant, or polytene, chromosomes of *Drosophila*. The banding patterns on these chromosomes have been used in studies ranging from molecular genetics to population genetics. Despite the usefulness and importance of the banding patterns, the causes of banding remain a mystery. Recent studies by Dr. Charles Laird have shed not only light, but also electrons, on the problem.

In 1881, G.G. Balbiani discovered sausage-shaped structures in the nuclei of secretory cells of *Diptera* (two-winged flies). He did not recognize them as chromosomes, and his report remained buried in the literature until 1933 when Painter, Heitz, and Bauer rediscovered them. Their studies showed that in secretory tissues (Malpighian tubules, gut, footpads, and salivary glands) of Dipterans, the chromosomes replicate many times without actually separating into chromatids. The process (endomitosis) gives rise to polytene chromosomes, which when stained with various dyes, have characteristic stripes which vary in size and morphology. The dark stripes of the polytene chromosomes are called bands; the light areas are called interbands.

Despite intensive studies over nearly 50 years, the underlying structure and significance of banding are still unknown. Conventional wisdom up to now has suggested that polytene chromosomes are made up of 1000-4000 complete replicates of each chromosome and that the bands are related to packaging of the genetic material. That is, the dark-staining bands have been thought to contain more tightly packed chromatin than do the pale interbands.

In recent years, however, a paradox has arisen. Estimates based on the staining properties of the interbands suggest that they are 3 to 5 percent DNA and that the ratio of DNA length to chromatin length (packing ratio) is about 3.5. These studies were based on light microscopy. However, other detailed microscopic studies have shown that interbands are composed of thick chromatin fibers which contain a large fraction of DNA and usually have a packing ratio of 20 to 56. This inconsistency has led Dr. Laird to reexamine some of the basic assumptions and interpretations regarding polytene chromosome structure.

Dr. Laird used high voltage electron microscopy to determine the density of the DNA along the length of unstained polytene chromosomes. His results, added to previous data, led him to propose an entirely new structure for polytene chromosomes. He suggests that interbands have fewer DNA copies than do the bands; that is, they are reduced in polyteny. Thus, according to Dr. Laird, the polytene chromosome is one DNA fiber, parts of which are replicated many times (bands) and parts of which are less replicated (interbands).

According to Dr. Laird, the junctions of bands and interbands are DNA replication forks, and the variations in DNA density are due to variations in polyteny along the length of the chromosome. Thus, although bands contain more DNA copies than do interbands, the percentage of DNA with respect to total mass in bands and interbands is about the same. Earlier estimates of 3 to 5 percent DNA content in interbands depended largely on the light microscope and on a series of assumptions about polytene chromosome structure that are probably inaccurate. The old picture of the polytene chromosome with many chromatids lying side by side now appears incorrect. It is replaced by a picture of the polytene chromosome which replicates differentially along its entire length. New technology utilizing the electron microscope allows more accurate measurements of DNA density and more detailed pictures of polytene chromosome structure.

If Dr. Laird's postulate is correct, our understanding of polytene chromosome structure and function will be sharply revised. Past studies on polytene chromosomes have been extremely valuable in understanding transcription and gene organization. Our knowledge of the importance of chromosome rearrangements (deletions, insertions, duplications, inversions, and translocations) came directly from studies on Drosophila polytene chromosomes. The techniques developed to study banding in these chromosomes have recently been put to use in human genetics. Preparations of human chromosomes can now be made in the laboratory and stained so that bands are revealed. The banding patterns can be used (much as Drosophila banding has been used) to study the relationships of chromosome organization to disease, the changes in gene expression throughout development, and the evolution and migration patterns of populations. Dr. Laird's hypothesis also suggests that Drosophila polytene chromosomes may be useful for studying DNA replication. Replication origins, replication forks, and replication enzymes should be abundant in polytene chromosomes. Thus, the system may find further usefulness as a model for studies on DNA replication.

"Research on Heat Shock Proteins Heats Up"

R01 GM 22274-07 (Meselson, M), Harvard University and R01 GM 25874-04 (Lindquist, S.), University of Chicago

Drosophila cells contain a small set of genes which are rapidly and vigorously activated by mild heating (a shift from 25° to 36° C). This "heat shock" response is a remarkably powerful model system for studying the organization of eukaryotic genes and the regulation of their expression. Virtually every tissue or cultured cell line from Drosophila responds in an identical manner. Within a few minutes after the heating, transcription is curtailed at most of the previously active loci and is initiated at a small number of new chromosomal sites. Protein synthesis is quickly redirected from the spectrum of proteins characteristic of that particular tissue to the production of a small number of new, so-called "heat shock" proteins. The activation of genes by heat treatment can be visualized cytologically in the puffs which form at specific sites on polytene chromosomes.

Recently, striking parallels have been found between this response in Drosophila and similar induction in a remarkably broad range of other eukaryotes. Proteins produced by heat treatment have been reported in cells from sea urchin embryos, Chinese hamster ovaries, chick fibroblasts, slime molds, yeast, and Tetrahymena. The physical properties of the induced proteins are very similar in all these organisms. In virtually every species investigated, the major protein induced has a molecular weight of approximately 70,000 daltons, and in organisms as diverse as fruit flies and slime molds, the proteins have the same isoelectric point and similar electrophoretic properties. Thus, the heat-shock response in Drosophila may prove to be a good model for eukaryotic stress-induced responses in general.

The heat shock response offers many advantages as a model system for studies of gene structure and expression. The induction of new protein synthesis following heat shock is unusually rapid, easily observed, and reproducible. The response provides an opportunity to study both gene activation and gene repression and is particularly useful in studies of translational control. Studies on this topic have been difficult for a variety of reasons, and few clear examples of translational control mechanisms which are selective for specific products have been discovered.

Dr. Susan McKenzie Lindquist has contributed significantly to the early description of the heat shock response in Drosophila. As a student of Dr. Matthew Meselson, Dr. Lindquist first mapped messages for the heat shock proteins to chromosomal sites. Dr. Lindquist's work has continued along several lines.

She performed a detailed analysis of the patterns and kinetics of protein and messenger RNA (mRNA) synthesis in Drosophila under a variety of different induction conditions. Although relatively routine, this work provided a thorough knowledge of the details of cellular dynamics useful for future experiments. For example, it provided a wealth of practical data concerning the exact culture conditions which yield maximum synthesis of mRNA's, data which are valuable in evaluating the response of heat shock mRNA's. It also provided some clues about how the response might be regulated.

While the heat shock response has often been characterized as the strictly coordinated induction of a small set of proteins, Dr. Lindquist has found that the synthesis of these proteins and their mRNA's can be individually modulated to a great extent. The induction of the separate molecular species must therefore be individually regulated to a considerable degree.

Another aspect of Dr. Lindquist's work has centered around the translation of the heat shock proteins. Pre-existing messenger RNAs are not degraded following heat shock. It was a surprise to observe this phenomenon because it had been thought that messenger RNA's are labile and rapidly degraded. Dr. Lindquist confirmed her observation in a variety of ways. (1) In one set of experiments, cells were heat shocked and then returned to 25°C for recovery. The rate of restoration of the full spectrum of normal protein synthesis was the same in the presence or absence of actinomycin D. Since actinomycin D blocks the transcription of new messages, translation must have occurred using mRNA molecules which were retained during the heat shock. (2) In a second set of experiments, the total cellular RNA complement was isolated from cells before, during, and after heat shock treatment, and the RNA was subjected to isoelectric focusing. The RNA's were blotted onto nitrocellulose in the presence of particular message sequences (such as those for actin). Sequences complementary to normal cellular messages were clearly detectable after heat treatment. (3) In another set of experiments, Dr. Lindquist attempted to assay messages by isolating them from heat shocked cells and translating them in vitro. Normal cellular protein synthesis does not come to a complete halt during these in vitro experiments but continues at approximately 10 percent of its normal level. Recovery of translational products in the in vitro system could therefore be assayed quantitatively. Dr. Lindquist was able to demonstrate translational activity in RNA's isolated from these heat shocked cells. The patterns of synthesis were very different from those obtained with polysomal RNA from control cells, and efficiencies were much lower. The observation suggests that after heat shock, Drosophila cells may still contain large quantities of cytoplasmic RNA's but that these are not translated efficiently in vivo. On the other hand, the messages might be physically altered either reversibly or by a copurifying RNA which is subject to translational control.

One of Dr. Lindquist's most interesting findings has been that Drosophila heat shock proteins migrate to the nucleus during the heat treatment and bind to the chromosomes. This finding was unexpected and is particularly interesting for its implications concerning control. Dr. Lindquist discovered this phenomenon when she was working with cells in which she had produced an aberrant heat shock protein containing substituted amino acids. The analog-substituted proteins were unable to enter the nucleus, presumably as a result of their larger size, and accumulated as a ring around the nuclear membrane. Active processes insert protein into cell membranes. Scientists have speculated that nuclear proteins enter by passive diffusion. Dr. Lindquist does not know whether the mechanism she observes represents a special emergency adaptation for rapid transport of heat shock proteins or a more general cellular process to transport large proteins into the nucleus. Investigations into this phenomenon should provide insights not only into the heat shock response but also into the basic problems of intracellular recognition and localization of proteins.

Dr. Lindquist has also made the observation that the heat shock proteins are required for recovery of the cell from heat shock. Additionally, she has found good evidence that autoregulation occurs in the heat shock response. That is, once the heat shock protein synthesis is induced it continues until some "sufficient" quantity of functional heat shock protein has been produced --even if the temperature is shifted back down.

Recognition that the heat shock response is present in virtually every eukaryote which has been examined exacerbates the chief frustration of working with this response -- that there is no clear understanding of what heat shock proteins do. However, studies of the heat shock response may uncover some more general cell mechanisms. This likelihood is supported by the observation that the puffs on polytene chromosomes of Drosophila cells following heat shock also occur when cells are treated with inhibitors of oxidative phosphorylation. This observation suggests that the response may be the cell's attempt to cope with changes in respiratory metabolism and the electron transport system.

Recent reports suggest that malignant cells appear to be less resistant to mild heat treatment than are normal cells. This finding has led to the use of hyperthermia as a treatment for some tumors. Thus the "heat shock response" has led both to the investigation of some fundamental questions concerning cell function and to a consideration of possible future medical applications.

"Developmental Genetics of Drosophila: Chorion Genes Amplify our Knowledge; and Gene Regulation by DNA Binding Proteins"
R01 GM 27875-03 (Spradling, A.), Carnegie Institute of Washington, D.C., and
R01 GM 24764-04 (Garen, A.), Yale University

The development of a comparatively simple organism such as Drosophila involves an incredibly complex sequence of events that is genetically programmed to occur in a precise temporal and spatial order. Nuclear transplantation experiments have shown that most, and probably all, cells in an organism contain virtually the same genetic information. This conclusion has profound significance because it follows that cell differentiation and specialization during development depend on selective expression of genetic information, which in the Drosophila genome consists of about 6000 genes. Scientists are only beginning to understand the molecular basis for selective gene transcription. Information about one important aspect of the problem, the organization of nucleotide sequences in the eukaryotic genome, is accumulating rapidly, because of the remarkable advances in methodology for analyzing complex DNA molecules -- notably mapping with restriction endonucleases, cloning DNA segments, and determining nucleotide sequences.

The complex processes underlying development are most readily studied by focusing on the functioning of specific genes. A major question concerns the mechanisms involved in the developmental control of gene expression. Currently, many genes are being intensively studied in an effort to shed light on this issue.

Recent work in the laboratory of Dr. Allan Spradling suggests that the process of eggshell (chorion) synthesis during the late stages of oogenesis in Drosophila is an example of a developmental process which is amenable to detailed analysis at many levels. This complex process provides an opportunity to analyze the functioning of a set of genes, beginning with their activation at specific developmental stages, the processing and translation of their primary gene products in the follicle cells, and finally the secretion of the chorion proteins and their assembly into an ordered macromolecular structure -- the eggshell. Specific mutations affecting structural genes involved in chorion production are readily obtainable; these give rise to biochemically and morphologically abnormal eggshells.

The focus of these studies has been narrowed somewhat due to an interesting recent finding. All the major chorion genes undergo a 12- to 60-fold differential DNA replication as part of their normal developmental programs. This is the first documented case of gene amplification involving protein-coding genes; it may be of fundamental importance in understanding their program of developmental expression. Therefore, much effort has concentrated on understanding this process. Specifically, a current goal has been to determine the molecular mechanisms by which the chorion gene clusters replicate differentially. It was also desirable to measure as accurately as possible the transcriptional programs of the four major chorion genes during development.

To study these questions, specific DNA probes were necessary. Stretches of Drosophila chromosomal DNA, including and adjacent to the major chorion genes, were cloned. In the aggregate, these fragments were some 300,000 nucleotide base pairs long. The following properties of the amplified DNA molecules were determined: (1) amplified sequences are present in large molecules (greater than 50 kb); (2) both strands of DNA are amplified equally; (3) chromosomal sequences flanking the genes are also amplified but to a lesser extent; (4) the magnitude of amplification is maximal in the coding regions, and decreases continuously as the distance from the genes increases. The total length of DNA showing any detectable amplification is about 100 kb on the X chromosome and 150-200 kb on the third chromosome.

Although making significant contributions to the molecular analysis of developmental events, even the most detailed sequence information cannot provide a full explanation for gene regulation. It is also necessary to consider other factors, such as temperature, the action of chromosomal proteins and of hormonal signals, and chemical modifications of bases in DNA and in the processing of messenger RNA.

Dr. Garen's studies on regulated gene expression in Drosophila are focused on six genes expressed specifically in larval fat bodies during the third-instar stage. Clones containing the coding genes for each of the six polypeptides have been isolated. Each of the six genes maps at a single site and appears to be present in a single copy. All are genetically unlinked. The occurrence of a coordinately regulated family of dispersed genes provides a favorable opportunity for molecular analyses of the regulatory mechanisms. Expression of several of the fat body genes is induced by ecdysone, a steroid hormone which has been shown to have an essential role throughout Drosophila development. In progress are genetic and molecular studies of the regulation of transcription of the ecdysone-inducible genes, and of other genes that are apparently not ecdysone-inducible in the fat body. Dr. Garen believes that a principal mechanism for developmentally regulated gene expression is the interaction of regulatory signals, particularly hormones and DNA-binding proteins, with the genome, in which the primary organization of the DNA remains constant at all stages.

The studies of Drs. Spradling and Garen focus on very different molecular aspects of the regulation of development. Spradling's studies emphasize changes in genome structure due to differential gene amplification. By contrast, Garen's studies focus on the interaction of hormones and other factors and picture a relatively more stable genome. It is clear from these diverse approaches and findings that the mechanisms of development are proving to be highly complex and subject to variable influences.

"Nomadic Genes in Drosophila"

R01 GM 25103-04 (Young, M.), Rockefeller University, and R01 GM 29097-01 (Strobel, E.), Purdue University

In Drosophila, more than one-third of the genome consists of DNA sequences which are repeated many times and which are interspersed among the single copy sequences. These repetitive sequences fall into two classes -- those that are highly repetitive (19 percent of the genome) and those that are moderately repetitive (16 percent of the genome). Highly repetitive sequences, which are reiterated approximately 24,000 times, are simple and short (5-378 bases) and probably are not transcribed into RNA. In contrast, moderately repetitive sequences are present at an average of 10-100 copies per haploid genome. Members of these repeat families are large (0.15 to 13 kilobases) and are dispersed throughout the genome. They are thought to be templates for messenger RNA (mRNA).

The arrangement and function of the moderately repetitive sequences in *Drosophila* constitute a major research interest of NIGMS grantee Dr. Michael Young who made the surprising discovery that much of the moderately repetitive DNA is nomadic. That is, it occupies no fixed positions on chromosomes.

Initially, Dr. Young prepared plasmids containing DNA sequences from randomly sheared *D. melanogaster* DNA. He selected plasmids which contained moderately repetitive DNA sequences and demonstrated that the sequences were located at many sites on the chromosomes of a particular strain. Radioactively labeled DNA from these plasmids was then hybridized *in situ* to polytene chromosomes of two genetically different strains of *D. melanogaster* in order to map the locations of the moderately repetitive sequences. Dr. Young found that the hybridization patterns in the two strains were different. Additional patterns in other strains confirmed this finding. Extrapolation from these results showed that approximately 15 percent of the total nuclear DNA is found in different chromosomal locations in closely related strains of *D. melanogaster.* This led Dr. Young to conclude that nearly all the moderately repetitive DNA is nomadic.

Dr. Young's laboratory investigated in greater detail the properties of one moderately repetitive nomadic gene family, named *copia*, which is composed of 20 to 30 chromosomally dispersed members. The coding regions of the *copia* genes are approximately 5 kilobases in length and are generally well-conserved throughout the genome, although several variants with deletions have been identified. Certain chromosomal locations are preferred sites of insertion of *copia* and other nomadic genes. However, the reasons for the preferences are unclear; the integration sites do not, for example, have the same DNA sequences. In this respect, the insertion of *copia* into the *Drosophila* genome resembles that of transposable elements in bacteria.

The properties of *copia* are also remarkably similar to those of certain integrated retroviral DNA's, and Dr. Young envisions that nomadic DNA may replicate and integrate in a manner similar to that of retroviral DNA. Both nomadic DNA and proviral sequences occupy a variety of positions in the host chromosomes, and they are structurally similar. Integration of the proviral DNA into the genome of newly infected cells occurs through reverse transcription of an RNA intermediate. Interestingly, RNA complementary to *copia* is enriched in nuclei, and the cytoplasmic *copia* RNA is not associated with polyribosomes. This unusual distribution of RNA raises the possibility that it may serve as a structural intermediate in the process of sequence integration.

Dr. Young recently constructed a strain of *D. melanogaster* with only 7 copies of *copia* rather than the 20 to 30 copies found in the wild type. Surprisingly, these "copia-reduced" flies had normal viability, fertility, and external morphology. In addition, the related species *D. simulans*, which contains one-sixth as many *copia* sequences, interbreeds with *D. melanogaster* to form viable, though sterile, offspring. These observations imply that the nomadic sequences are not essential to the organism's survival.

Nomadic genes may increase genetic diversity in populations by facilitating the acquisition of new genes which could not enter the genome by other recombination mechanisms. Conversely, if a population were especially well-adapted to its niche, genetic variability introduced by mutations or interbreeding would be undesirable. Nomadic genes could restrict variability by forming barriers to gene flow between populations.

Nomadic genes might affect expression of neighboring genes. NIGMS grantee Dr. Edward Strobel has studied the chromosomal arrangements of *copia* and other dispersed repeated sequences. He has shown that insertion of *copia* within or near a specific genetic locus can profoundly affect the expression of that locus. For example, the locus which causes the unstable mutation *white* contains a *copia*-like sequence. In wild type revertants, this insert is deleted. Insertion of nomadic sequences at other positions might be involved in control of gene transcription or in the regulation of developmental changes.

Research on nomadic genes, like that on other transposable elements, has raised more questions than it has answered. The presence of transposable elements in eukaryotic cells is posing many interesting biological questions about genetic recombination, gene expression, and even evolution. *Drosophila* is turning out to be an excellent model system for these studies.

"*Abnormal Abdomen Syndrome in Drosophila mercatorum: Molecular, Developmental, and Population Studies*"
R01 GM 27021-02 (Templeton, A.), Washington University, St. Louis

The complete understanding of a biological system requires studies at several levels of biological organization. Dr. Alan Templeton has successfully exploited a syndrome known as abnormal abdomen (*aa*) which is found in natural populations of the fruit fly, *Drosophila mercatorum*, in studies ranging from molecular to population genetics. The syndrome, which affects many aspects of development and aging, depends on a major gene on the X-chromosome, as well as on minor genes which modify the expression of the major gene. The *aa* syndrome is normally expressed only in females, but one of the modifier genes on the Y-chromosome allows *aa* to be expressed in males whose X-chromosome carries *aa*.

Because some of the traits associated with *aa* resemble known ribosomal deficiency traits in *D. melanogaster*, Dr. Templeton's molecular studies focus on the ribosomal RNA (rRNA) in the *aa* flies. The two major rRNA's of *D. mercatorum*, 28S and 18S, are coded by tandemly duplicated DNA units located on the X-chromosome, Y-chromosome, and two autosomes. By recombinant DNA techniques, he discovered that X-chromosomes bearing *aa* have DNA sequences of six to seven kilobases which are inserted into the coding region of most of the 28S genes of the X-chromosome. The insertions are not present in the 28S genes of non-*aa* flies. Moreover, in males expressing *aa*, all of the rRNA on the Y-chromosome is deleted. Other experiments showed that the insertions on the X-chromosome of *aa* flies block transcription of the X-linked genes for rRNA, leading to a ribosomal deficiency. The deficiency can be compensated for in males by the rRNA genes normally found on the Y-chromosome; but if the Y-linked genes are deleted, the males will also display the *aa* syndrome. The molecular basis for *aa*, therefore, lies in insertions and deletions of genes coding for rRNA on the sex chromosomes.

Since all protein synthesis occurs on the ribosomes, it is not surprising that *aa* has many effects on development. Relative to normal flies, some tissues in *aa* flies develop more rapidly or more slowly, causing *aa* flies to have novel sets of concurrent traits and greatly altered aging patterns. For example, larval aging is slower in *aa* flies than in normal flies, but adult aging is more rapid. As a result, adult *aa* flies become sexually mature twice as fast as normal but are very short-lived.

One hypothesis to explain this developmental pattern is that tissues which must synthesize large quantities of a particular protein in a short period of time will be especially sensitive to the effects of the ribosomal deficiency produced by *aa*. Dr. Templeton examined one such protein, juvenile hormone-specific esterase, in *aa* and non-*aa* flies. The larval phase of flies is maintained by the presence of juvenile hormone. For normal metamorphosis to occur, juvenile hormone must be rapidly degraded by the esterase. Although esterase proteins in both strains were structurally very similar, it took longer for esterase activity to reach normal levels in *aa* flies. The continued presence of undegraded juvenile hormone changes the rate of development, especially in the cells destined to become abdominal segments. Flies with *aa*, therefore, have abdomens with an undeveloped cuticle rather than the normal adult cuticle.

The changes in developmental rates associated with *aa* directly alter the life history of *D. mercatorum*. The life-history, which includes such features as survival, fertility, and fecundity, determines the fitness of the flies. Dr. Templeton has performed field studies to investigate the evolutionary significance of *aa*. These studies show that *aa* is adaptively important under conditions of low humidity where death occurs through desiccation. Flies with *aa* are slightly more sensitive to desiccation than are non-*aa* flies. However, the earlier sexual maturation of *aa* females offsets this disadvantage and gives them more time to lay eggs, giving them an adaptive advantage. In areas of higher humidity non-*aa* flies, with their longer life span, have greater fitness. As a result, in the field, *aa* flies are found in relatively higher frequencies during droughts or in regions which are normally dry, but are rare at more humid sites. Under certain environmental conditions, therefore, this gene helps the population of *Drosophila* to survive. This situation is similar to the well-known case of sickle cell anemia in humans, in which carriers of the disease are resistant to malaria. The abnormal abdomen syndrome in *Drosophila* promises to be an excellent model system for studying interactions between genes and the environment and their evolutionary consequences.

2. NEW INSIGHTS INTO GENE ORGANIZATION

Introduction

This year, as in the past several years, biomedical scientists supported by the Genetics Program of NIGMS have made significant advances in understanding the molecular mechanisms underlying many cellular processes, including DNA replication and the transcription and translation of gene products; stabilization, differentiation and regulation of gene expression; and mutation, repair and evolution. Advances in these areas are in a large part due to an increased knowledge of the structure of the genome. In fact, so many exciting aspects of genomic structure have been reported during the past year that we have decided to devote a major portion of this Annual Report to highlights of studies on genomic structure, particularly as they relate to our understanding of cellular processes.

Genome refers to the factors which determine the hereditary characteristics of a cell. The term genome was originally meant to include only the DNA. It has gradually acquired a broader meaning because it is now recognized that the expression of genes coded for by particular sequences of DNA is dependent on many other molecules. The functional genome may be thought of as consisting of several levels of molecular complexity. At the most basic level, nucleotides are arranged in specific sequences to form a molecule of DNA which may then be folded in ways that can affect gene expression. At the next level of complexity, genes are arranged along the DNA strand. The DNA, in turn, interacts with a variety of structural and regulatory proteins and with RNA to form the next level of structure known as chromatin. Chromatin is packaged into still more complex structures, the chromosomes, which are thought to occupy specific locations in the nucleus.

The review of the research that follows is arranged according to the levels of organization of the genome. At the end of the section we relate some studies on possible future applications derived from our improved understanding of genomic structure.

DNA Structure

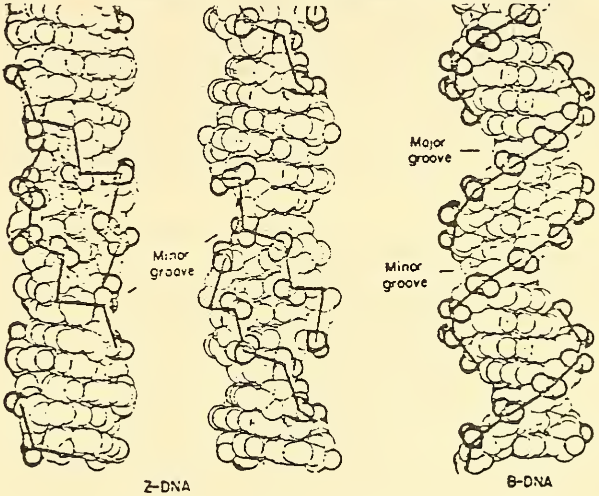
"Right- and Left-Handed DNA are Right in the Chromosomes"

R01 GM 30543-01 (Dickerson, R.), University of California, Los Angeles, and R01 GM 21874-07 (Pardue, M.L.), Massachusetts Institute of Technology

Recently, Dr. Richard Dickerson completed X-ray crystallographic studies of the structure of an oligonucleotide. The dodecamer CGCGAATTCGCG, which was synthesized by Dr. Koiichi Itakura (City of Hope Research Institute, GM 24393) formed the expected right-handed helix (called B-DNA) and is the first B-DNA to be structurally characterized. It is also the first characterized structure of a complete turn of a helix.

A related, but more surprising, development was also published this year. A paper co-authored by NIGMS grantee Dr. Mary Lou Pardue reported that a newly discovered form of DNA occurs naturally in chromosomes. This intriguing discovery raises the possibility that this form of DNA may play an important role in regulating gene activity.

This story began ten years ago when synthetic DNA with alternating bases of guanine and cytosine was found to produce a peculiar circular dichroism spectrum when put into solution with a high concentration of salt. The reason for this spectrum went unexplained until 1979 when Dr. Alexander Rich and co-workers at the Massachusetts Institute of Technology and the University of Leiden in the Netherlands performed X-ray diffraction studies on synthetic DNA. They found that DNA with alternating purine and pyrimidine bases can, under particular conditions, undergo a transition from its usual right-handed helical twist to a left-handed twist, which was the form observed in the circular dichroism spectrum. In the left-handed form guanine bases rotate about the glycosidic bond, causing the sugar-phosphate backbone to follow a zigzag course. Hence, the left-handed DNA was named Z-DNA (see figure).



Van der Waals sideviews of Z-DNA and B-DNA

This observation might have remained just a chemical curiosity, except that Dr. Rich's group followed these studies with experiments to investigate whether Z-DNA has any relevance to biological systems. Their first step was to produce anti-Z-DNA antibodies in rabbits and mice. Because of their specificity, antibodies have been used to determine the distribution of many molecules in biological systems. Dr. Rich's group, collaborating with Dr. Pardue, used anti-Z-DNA antibodies to detect Z-DNA in Drosophila polytene chromosomes. The banding pattern of each chromosome is constant from nucleus to nucleus and from individual to individual. Polytene chromosomes provide an excellent system for studying the conformational states of specific regions of the chromosome at the time when the DNA is synthetically active.

Salivary glands of Drosophila larvae were squashed on a glass slide and freeze-dried. The polytene chromosomes, which are well spread by this procedure, were treated first with the rabbit antibodies to Z-DNA. After washing off unbound antibodies, the chromosomes were treated with fluorescently labeled goat antibodies which reacted with the bound rabbit antibodies. By this technique, which is known as indirect immunofluorescence, the fluorescently labeled second antibody could be observed by fluorescence microscopy.

The anti-Z-DNA antibodies stained the polytene chromosomes in a very distinct way. By matching the fluorescent preparations with the banding patterns observed by phase microscopy, the investigators found that the antibodies reacted specifically with the interband regions.

Caution must be exercised in drawing conclusions from immunofluorescence data since artifacts are common. Recognizing this, the investigators performed extensive controls in which chromosomes were prepared by a variety of methods, all of which produced similar results. They also demonstrated that immunofluorescence could be prevented by preincubating the anti-Z-DNA antibodies with synthetic Z-DNA but not with native or denatured DNA from Drosophila embryos. These results led to the conclusion that Z-DNA is a naturally occurring form of DNA and that it is either more abundant or more accessible in the interbands than in the bands of chromosomes.

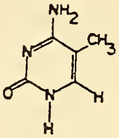
At physiological salt concentrations, Z-DNA is less stable than B-DNA. However, studies on synthetic DNA show that there is a reversible equilibrium between the two forms when the molecule has the alternating sequence of guanine-cytosine. Stabilization of Z-DNA in chromosomes might be achieved by supercoiling, by binding to proteins which are specific for the Z conformation, by binding to specific ions, or by modifications such as methylation of cytosine. Interestingly, methylation of cytosine residues is also correlated with gene activity (see next highlight).

Sequencing of DNA has demonstrated the existence of biologically active regions with alternating guanine and cytosine residues. Since active regions are probably less tightly coiled than are inactive regions, it is possible that transitions between the two forms of DNA containing the guanine-cytosine sequences could be related to the activity of genes. What began as a chemical oddity might turn out to be an important feature for regulating gene transcription or replication.

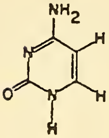
"Gene Activity is Related to Patterns of DNA Methylation"
R01 GM 20483-08 (Cedar, H.), Hebrew University-Hadassah Medical School, Jerusalem, and R01 GM 26176-03 (Weintraub, H.), Fred Hutchinson Cancer Center, Seattle

DNA is a polymer composed of four nucleotide bases: the purines, adenine and guanine; and the pyrimidines, thymine and cytosine. In eukaryotic DNA, a small proportion of the cytosine residues are methylated by an enzymatic process in which the methyl group from S-adenosylmethionine is transferred to position 5 of cytosine (see figure). Addition of the methyl group, which occurs after DNA synthesis, changes the conformation of the DNA in the region of the methylcytosine (m^5C) residue and probably modifies the interaction of proteins with DNA.

5-Methylcytosine
(m^5C)



Cytosine
(C)



Most m^5C residues are located adjacent to guanine (G) residues. When a complementary strand of DNA is synthesized, cytosine pairs with guanine, forming a site where m^5CG is paired with GC. Methylation enzymes (methylases) recognize this site on the original DNA strand and quickly add a methyl group to cytosine on the complementary strand, leading to a fully methylated site:

$$-m^5CG- \xrightarrow[\text{synthesis}]{\text{DNA}} \begin{array}{c}-m^5CG-\\ -GC-\end{array} \xrightarrow{\text{methylase}} \begin{array}{r}-m^5C\ \ G-\\ -Gm^5C-\end{array}$$

Likewise, when a cytosine residue on the original strand is unmethylated, the cytosine complementary to the adjacent guanine remains unmethylated. By maintaining this symmetry, the methylation pattern is inherited by the daughter cells, resulting in tissue-specific methylation patterns.

It has been possible to identify and quantitate m^5C in DNA with a high degree of sensitivity by means of restriction enzymes. The endonucleases HpaII and MspI both cleave double-stranded DNA between cytosine and guanine when these residues occur in the sequence CCGG. While MspI cleaves this sequence whether or not cytosine is methylated, HpaII cleaves only the unmethylated sequence. The locations of m^5C in CCGG sequences can be determined by comparing the DNA fragments that are produced after digestion with either HpaII or MspI.

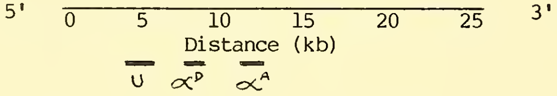
Results from such studies performed in the last two years showed that genes which are actively transcribing RNA are undermethylated compared to genes which are inactive. For example, the degree of methylation of cytosine in the β-globin gene region of adult erythrocytes is lower than in the comparable genes of oviduct or brain which do not synthesize β-globin.

Drs. Naveh-Many and Cedar recently examined in greater detail the relationship between gene activity and methylation patterns and demonstrated that undermethylation is a general phenomenon in all actively transcribed genes. As an indicator for gene activity they took advantage of the fact that transcriptionally active regions of DNA are much more sensitive than inactive regions to the enzyme deoxyribonuclease I (DNase I). The increased sensitivity to DNase is due to altered chromatin structure in the region of active genes.

Drs. Naveh-Many and Cedar treated nuclei of cells with DNase I to produce "nicks" in the DNA. The nuclei were then incubated with DNA polymerase in the presence of radioactively labeled nucleotides, a procedure known as nick-translation. Because DNase I preferentially attacks active regions, these genes were specifically labeled. The DNA was then treated with MspI or HpaII, and the size distribution of fragments was determined after electrophoresis. Labeled, active DNA had an unmethylated HpaII sensitive site every 3500 base pairs, while total erythrocyte DNA had this site only once in every 7000 base pairs. As expected, the number of MspI sites was equal in total DNA and in labeled, active regions, since MspI cleaves both methylated and unmethylated CCGG sequences. These results demonstrated that active regions as determined by DNase I sensitivity are undermethylated.

This conclusion was confirmed by a more direct approach. DNA which was labeled by nick-translation was treated to remove chromatin proteins and was allowed to hybridize with total poly(A)-RNA. Active, but not inactive, DNA sequences should hybridize with this RNA. Drs. Naveh-Many and Cedar found that the hybridized (active) DNA was strikingly undermethylated compared with unhybridized (inactive) DNA.

Drs. Weintraub, Larsen, and Groudine have also been interested in the relation between gene activity and structural changes in chromatin. They specifically examined the α-globin gene cluster which contains three α-globin genes (see figure) whose expression is developmentally regulated. Erythrocytes from 14-day chick embryos express α_A and α_D genes; erythrocytes from 5-day embryos also express the U gene. Dr. Weintraub's group mapped the MspI sensitive sites in the α-globin gene cluster and demonstrated that in erythrocytes of 14-day embryos the genes for α_A and α_D were undermethylated compared to those of other tissues. They also found that the spacer region between the genes and the region immediately after the genes (at the 3' side) were undermethylated but that the region preceding the active genes (at the 5' side) was methylated. The coding and spacer regions were also DNase sensitive while adjacent regions had intermediate sensitivity.



In erythrocytes of 5-day embryos the U gene is undermethylated and DNase sensitive. By 14 days, however, when the gene is no longer active, all MspI sites are fully methylated and the DNA is not sensitive to DNase I. This direct demonstration that methylation and DNase sensitivity change during differentiation is a significant advance in understanding the regulation of differentiation at the molecular level.

Other experiments by Dr. Weintraub's group and also by Drs. Jones and Taylor provide a possible explanation of how methylation may regulate differentiation. When mouse cells were treated for even a short time with 5-azacytidine, an analog of cytosine which cannot be methylated, they underwent differentiation into muscle, chondrocytes, and adipocytes. These changes in phenotype were related to undermethylation of the DNA.

The exact relationship between the methylation pattern of genes and their transcriptional activity is not understood. One current model proposed by NIGMS grantee Dr. Arthur Riggs (City of Hope Research Institute, Duarte, California GM 25825-12) and others is that in undifferentiated cells the DNA is fully methylated. During development, at the time of a critical DNA replication, sequence-specific proteins might inhibit methylation at particular sites. Alternatively, sequence-specific endonucleases could cause symmetrical excision of m^5C. Following repair in the absence of methylase the cytosine would remain unmethylated. Whatever the mechanism, the affected gene would be activated. Since the new methylation pattern is stably inherited, transient exposure to an inducing agent would lead to expression of the gene in all progeny cells. A mechanism for adding methyl groups de novo, if such an event occurs, has not been proposed. Further studies will be needed to characterize the factors which promote or inhibit methylation. Research in this area could go a long way toward explaining the mechanisms by which the interactions between cells and with extracellular matrix components control development.

Arrangement of Genes along DNA

"Overlapping Genes-- Strategy for Efficiency in a Small Genome"

R01 GM 27064-02 (Siegel, A.), Wayne State University, Detroit

Recent information about the organization of the genomes of higher organisms has suggested that the DNA contains extensive redundancies, non-coding regions, and other regions that, at first glance, seem inefficient. This structure may, however, provide certain advantages to the organism that make it worthwhile to maintain this extra DNA. For other organisms, such as viruses, evolution has led to a very different genomic structure. Research from the laboratory of Dr. Albert Siegel describes the extent to which viruses have increased the density of information stored in their nucleic acids.

Most cellular processes are carried on by proteins, whose amino acid sequence is encoded by the four nucleotides, A, C, G, and U, that make up RNA. The RNA itself is transcribed from the cell's DNA, which is made up of four nucleotides, A, C, G, and T. A linear DNA sequence of three nucleotides, called a codon, specifies each amino acid. The RNA sequence AAA, for example, specifies lysine, UUU specifies phenylalanine, and so on. Of the 64 possible combinations of 4 nucleotides taken 3 at a time, 61 specify amino acids. Three codons serve as signals to stop translation, and one codon, AUG, serves both as a signal to start translation and to code for the amino acid methionine.

Two specific aspects of the genetic code must be noted. First, the code is degenerate in that amino acids may have more than one codon. Leucine, for example, is specified by the UUA, UUG, CUU, CUC, CUA, or CUG codons. On the other hand, the amino acids methionine and tryptophan are each specified by only a single codon. Second, the code has a correct "reading frame" established by the position of the AUG start signal, and the code is read in a sequential, non-overlapping fashion like this:

$$\underbrace{\text{A U G}}\ \underbrace{\text{C C C}}\ \underbrace{\text{C G A}}\ \underbrace{\text{U U G}}\ \underbrace{\text{A U U}}\ \underbrace{\text{U A U}}$$

met - pro - leu - arg -ileu - tyr

and not like this

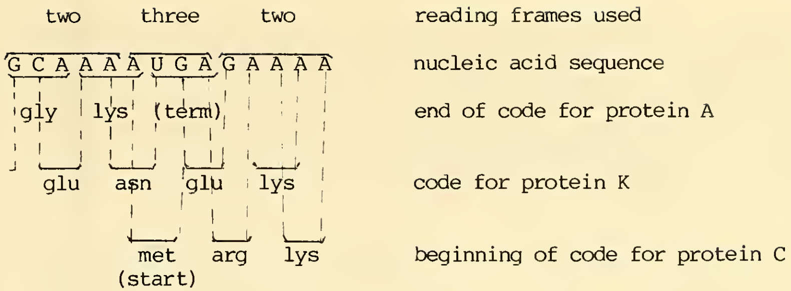
$$\underline{\text{A U G}}\ \text{C C C C G A U U G A U U U A U}$$

met
cys
ala
pro
etc.

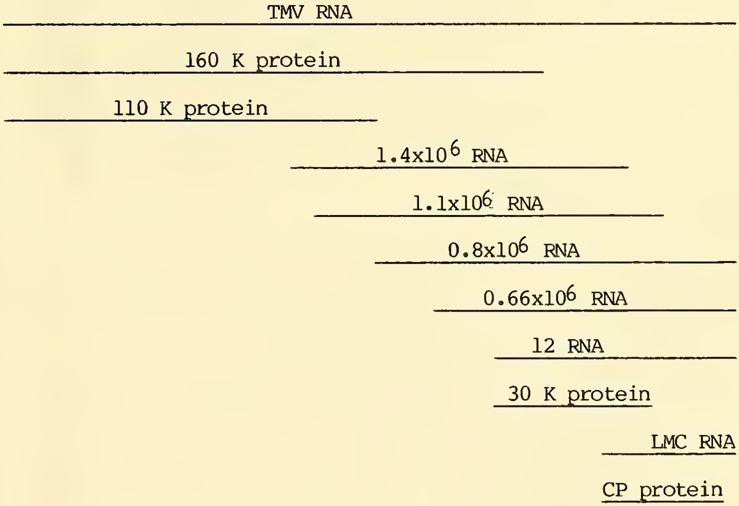
At present, only one reading frame is known to be utilized in coding sequences in the cell's own genetic material.

In viruses, however, the rule of only one reading frame does not always apply. Viruses are highly specialized and they employ the biochemical machinery of the hosts they invade for their energy metabolism and protein synthetic needs. They must, however, encode in their own nucleic acid specific proteins to ensure their own replication and packaging. Probably because of the limited space available for nucleic acid inside the virus' encasing protein coat, a number of viruses have developed overlapping genes. In some cases, two or even three different proteins are encoded in the same nucleic acid segment by utilizing more than one of its reading frames. Thus, a single nucleotide may be part of either one, two, or three codons, depending on whether one, two, or three of the available reading frames are used. Where overlapping genes occur, the genetic material is efficiently used. This, however, is done at a sacrifice in flexibility of choice in codon sequence because the codons used in a reading frame must not be read as terminator codons in another reading frame.

Despite predicted theoretical difficulties, a number of well established instances of gene overlap in viruses have recently been discovered. The first of these was found in a small bacterial virus called phi X 174. An example of a portion of its nucleotide sequence where two and even three reading frames are used is as follows:



Several other examples of multiple coding use have recently been found in the papovaviruses and the orthomyxoviruses. Dr. Albert Siegel has been studying the overlapping genes and their translation strategies in two plant viruses, the tobacco mosaic virus (cowpea strain) and the tobacco rattle virus. These viruses are of interest because they make use of at least two different types of gene overlap. Dr. Siegel has found that the well-studied tobacco mosaic virus has several heretofore unknown genes that can be accommodated on the viral genetic material only by gene overlap. This overlap may have at least two different expressions. One type is exhibited when the viral genome, which is composed of single stranded RNA, acts as a template. In this case there is a common start point for two different proteins. The translation of one protein ceases when it accumulates 1000 amino acids, whereas translation of the second continues until an additional 450 amino acids have been added. Thus, two proteins are generated, one larger than the other but both alike at one end. The other type of gene overlap results from another phenomenon. Genes on the tobacco mosaic virus RNA are generated from the virion RNA as illustrated below.



Two of these sub-genomic RNAs have been fairly well characterized; the smaller one (LMC) has been shown to code for the viral packaging protein (CP) and the larger one (I_2) to code for a protein of unknown function. The terminal coding sequence for the I_2 protein overlaps the beginning of the packaging protein gene. Other larger sub-genomic RNA species have been detected, and these probably contain coding sequences which overlap almost completely with that for the larger of the two proteins translated from the virion RNA.

The question of how widespread the phenomenon of overlapping genes may be and its significance for making efficient use of genetic material is under careful investigation in Dr. Siegel's and other laboratories. It is clear that the study of the organization and expression of genetic material in model systems has revealed unexpected phenomena such as overlapping genes. Viruses have proven to be excellent model systems for many studies, and in a number of instances have proven to be harbingers of genetic phenomena subsequently discovered in higher organisms. It is not yet known whether overlapping genes exist in higher organisms. However, the increased efficiency of this arrangement in small viruses may well be offset by the enhanced effect of mutagens or the necessarily tighter coupling of expression characteristic of overlapping genes.

"Gene Amplification by Illegitimate DNA Replication May Lead to Malignant Transformation of Cells"
R01 GM 26086-03 (Varshavsky, A.), Massachusetts Institute of Technology

Chemicals which promote tumors but are not carcinogenic by themselves increase the probability that a cell previously exposed to a carcinogen will become malignant. These substances include the potent 12-0-tetradecanoyl phorbol-13-acetate (TPA) which is isolated from the seeds of the croton plant, related phorbol esters, and possibly saccharin, cyclamate and phenobarbital.

In addition to its tumor-promoting activity, TPA has a variety of effects on cultured cells, some of which mimic effects of true carcinogens and mutagens. Like carcinogens, TPA alters cell shape, stimulates cell proliferation, reduces extracellular matrix production, induces plasminogen activator and ornithine decarboxylase activities, and increases sugar transport. In contrast to carcinogens, however, the effects of TPA are usually reversible after removal of the compound.

The mechanism by which TPA causes its diverse biological effects is not known. Dr. Alexander Varshavsky has recently put forth an intriguing new hypothesis for a genetic mechanism for tumor promotion. In eukaryotes, DNA replication is initiated at multiple sites. Except in a few special cases (such as in ribosomal gene amplification in amphibian oocytes or the formation of DNA puffs in polytene chromosomes of the insect *Rynchosciara*), each origin of replication initiates only one round of DNA synthesis at each cell cycle, and the probability of an extra ("illegitimate") round of DNA replication is low. Dr. Varshavsky has proposed that TPA may increase the frequency of illegitimate rounds of DNA replication in different chromosomal domains, including those containing transforming genes. The idea originated with the now-classic studies of Dr. Robert Schimke and colleagues (GM 14931-15) which demonstrated that cells develop resistance to the lethal effects of methotrexate, an inhibitor of the enzyme dihydrofolate reductase (DHFR). Resistant cells produce more DHFR because they contain more copies of the DHFR genes. These extra copies, which accumulate in the presence of methotrexate, probably result from illegitimate rounds of DNA synthesis, with each round producing an extra copy of the DHFR gene. The increased gene dosage leads to increased production of DHFR, which in turn confers a selective advantage on a cell when it is exposed to an otherwise lethal concentration of methotrexate. The amplified genes may exist as unstable extrachromosomal elements or may be stably incorporated into chromosomes.

To test the hypothesis that TPA facilitates gene amplification, Dr. Varshavsky measured methotrexate resistance in mouse cells grown in culture in the presence or absence of TPA. The frequency of methotrexate-resistant cells was increased 100-fold in the presence of TPA but not in the presence of the non-tumor promoting parent compound, phorbol. Several other compounds, including a weak non-phorbol tumor promoter, anthralin, and two hormones, vasopressin and insulin, also increased methotrexate resistance.

Next, Dr. Varshavsky measured the number of DHFR genes per cell in colonies resistant to methotrexate. To do this, he obtained from Dr. Schimke a segment of DNA complementary to the DHFR gene. This DNA probe, which was radioactively labeled, was hybridized to the DNA of methotrexate-resistant colonies. DNA from these resistant cells was more heavily labeled with the DNA probe, proving that the DHFR genes in these cells were amplified.

In the last several years, scientists have shown that certain transformed or malignant cell types contain more than the normal amount of a specific protein. By analogy with the amplification of DHFR genes by TPA, Dr. Varshavsky has postulated that tumor promoters may be involved by enhancing illegitimate replication of genes associated with transformation. This would lead to the synthesis of elevated levels of a protein (or proteins) required to maintain the transformed characteristics of a cell. The growth advantage of cells with increased doses of transforming genes would lead to the retention of these cells in a population.

Whether tumor promoters do, in fact, amplify transformation-associated genes as they do DHFR genes awaits a direct test. Dr. Varshavsky's current efforts are directed toward studying molecular mechanisms of the TPA effect on amplification.

"A Common Mother for All Laboratory Mice?"

R01 GM 21509-07 (Wilson, A.), University of California, Berkeley

Surprising results from a recent study by Drs. Stephen Ferris, Richard Sage, and Allan Wilson suggest that most inbred strains of laboratory mice are descended from a single female who lived some time between 1200 BC and 1920 AD.

This conclusion is based on an analysis of mitochondrial DNA (mtDNA) from inbred and wild strains of mice. Unlike nuclear DNA which is inherited from both the mother and father, mtDNA is inherited only from the mother through the egg cytoplasm. Because mtDNA in mice and certain other species is thought to evolve rapidly, it has become a favorite molecule in which to analyze evolutionary relationships among organisms. The more similar the nucleotide sequence, the more closely related are the organisms. Structural variations in DNA from different sources can be detected by treating the DNA with restriction endonucleases, enzymes that cleave DNA at specific nucleotide sequences. When enzymatic cleavage is followed by gel electrophoresis, the DNA fragments separate from each other on the gel. The extent to which DNA's are similar in nucleotide sequence can be estimated from the number of fragments they have in common.

Drs. Ferris, Sage, and Wilson found a high level of variation in mtDNA among 20 wild mice collected from North America, Europe, North Africa, and the Near East, and among five inbred strains that were established within the last 30 years from wild mice. In contrast, nine "old" inbred strains showed no variation in the mtDNA fragment pattern, despite the fact that 150 fragments were compared. Among the inbred strains studied were DBA, BALB/c, SWR, and C57BL/6J, all of which are commonly used in the laboratory.

The absence of variation in the maternally derived mtDNA among the old inbred strains contrasts with the large variations in nuclear DNA in these strains. To account for this, the authors suggest that a single female lineage founded all the old inbred strains and that nuclear DNA diversity among these strains is due to the mating of this lineage with diverse males.

The historical breeding records are incomplete, but what is known about the origins of the various old inbred strains is consistent with the idea of a single founding mother. The inbred strains could have arisen as late as 1920, the time at which these strains were first established, and when a female mouse of one strain may have inadvertently been bred with males from different strains. But it is also possible that the original mother lived as long as 3400 years ago when mice were first domesticated in China. Laboratory mice could be descended from these mice or from mice domesticated independently in Europe.

An alternate explanation for the lack of variation in the maternally derived mtDNA is that mice with a particular type of mtDNA had a selective advantage because it promoted survival in a domestic or laboratory setting. However, the selectionist explanation does not fit the observation that wild mice have given rise more recently to new inbred strains possessing other types of mtDNA.

There are a number of lessons to be learned from this study. The first is that laboratory mice are indeed different from wild mice, and that more studies on wild mice may be worthwhile. Second, one cannot equate data on genome diversity derived from mitochondrial and nuclear DNA analyses in forming conclusions on evolutionary relationships. Third, because of the homogeneity of mtDNA in laboratory mice, they may be valuable organisms in which to study nuclear-cytoplasmic interactions in development. And finally, the study draws attention to the possible inadvertent mixing of other supposedly distinct stocks.

Chromatin Structure: The Interactions of Proteins and DNA

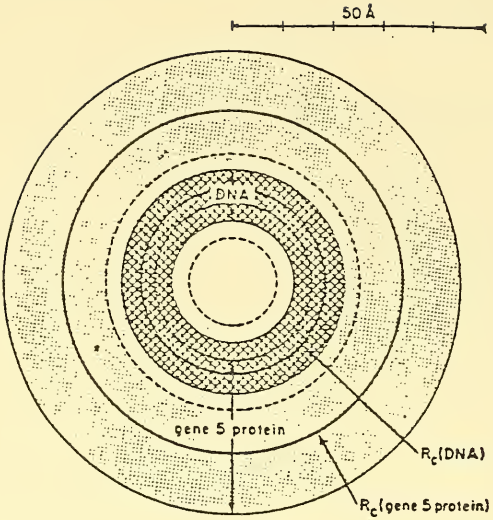
"Structure of a Complex of fd DNA and Gene 5 Protein"

R01 GM 19060-09 (Gray, D.), University of Texas, Dallas

One of the key issues in gene expression is the interaction of proteins with nucleic acids. In eukaryotes one cannot talk about the structure of the genome without including a discussion of the proteins which, together with nucleic acids, make up chromatin. The mechanism by which nucleic acids and the associated proteins can be wound up to form an exceedingly compact structure, but still relax sufficiently to permit replication, is a central unsolved problem in structural biology. At present, however, the complexity of the interactions between protein and DNA precludes the detailed examination of chromatin as a whole. Hence, most studies involving investigations of forces between proteins and nucleic acids have focused either on one specific aspect of chromatin structure or on simpler protein-nucleic acid systems.

Dr. Donald Gray, in collaboration with Dr. C. W. Gray (University of Texas-Dallas) and Dr. R. D. Carlsen (Brookhaven National Laboratory), has been studying the interaction of DNA and gene 5 protein from the filamentous bacteriophage fd. During replication, an intracellular complex is formed between the protein coded by the phage gene 5 and single strands of progeny DNA. On completion of the synthesis of a single-stranded viral DNA molecule, the DNA-gene 5 complex is transported to the bacterial membrane, and during the extrusion process the gene 5 protein is displaced by the coat protein subunits which reside in the membrane. The fd DNA-gene 5 protein complex, which is a key structure in this process, serves as a general model for studying DNA-protein interactions.

Dr. Gray's approach has been to use neutron diffraction to obtain the radial displacement of the DNA, relative to the protein, in the complex. Early results indicated that the neutron scattering data for the complex in solution agree with the model proposed from simple crystal X-ray diffraction data of McPherson et al. with the important exception of the position of DNA. The contrast dependence of the cross-sectional radius of gyration and the calculated maximum radius of the DNA backbone both indicated that the DNA was not on the periphery of the helical complex as proposed by McPherson. However, since DNA comprises only about 12 percent of the total mass in the complex, an unambiguous analysis of the DNA position was difficult. Dr. Gray was able to enhance the scattering of the DNA by replacing most (87 percent) of the hydrogens by deuterium. A series of careful measurements was then carried out on the reconstituted complex over a full range of solvent conditions. These results confirm Dr. Gray's earlier conclusion that the DNA component is near the center of the complex as shown in the figure below, and is not, as proposed by McPherson, a complex made up of two DNA single strands spooled around a spindle of gene 5 protein.



Cross section of the complex between DNA and gene 5 protein. R_C(DNA) and R_C(protein) are the radii of gyration at infinite contrast for the DNA and protein components. The cylindrical shell occupied by the DNA (crosshatched) extends from 11.8 to 21.8 Å, and the shell occupied by the protein (stippled) extends from 14.9 to 44.9 Å. The dashed rings show the uncertainty in the DNA shell due to the error of ± 3Å (± 1 standard deviation) in the determination of the maximum $\overline{R}_C(\overline{DNA})$.

Dr. Gray's measurements have important implications for the mechanism of viral packaging. The determination that DNA is near the center of the complex means that the DNA does not have to undergo a great radial displacement during its packaging into mature fd phage. Furthermore, in addition to providing an important piece of information which may lead to a better understanding of protein-nucleic acid interactions, Dr. Gray's work also emphasizes the care which needs to be taken in extrapolating X-ray results obtained in crystals to structures in solution.

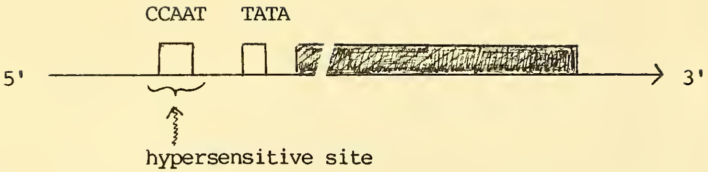
"DNase Hypersensitive Sites May Regulate Gene Activity"

R01 GM 30273-01 (Elgin, S.), Washington University, R01 GM 21514-07 (Gilbert, W.), Harvard University, R01 GM 26176-03 (Weintraub, H.), Hutchinson Cancer Center, and R01 GM 21574-06 (Worcel, A.), University of Rochester

The last ten years have seen tremendous advances in our understanding of the structure of chromatin, the DNA- and protein-containing material which constitutes the chromosomes. Biochemical analyses have characterized many of the proteins associated with the DNA, and electron microscopy has clarified the structural relationships between the DNA and proteins. Now, investigators have begun to examine how chromatin structure affects gene activity. One breakthrough was the finding by Dr. Harold Weintraub in 1976 that regions along the chromosomes which contain transcriptionally active genes are ten times more sensitive to the enzyme deoxyribonuclease I (DNase I) than are inactive regions. This finding suggested that active regions have a more relaxed structure which enhances their accessibility to the enzyme.

Several very recent publications demonstrate that, in addition to these sensitive regions, chromatin from a variety of species contains regions which are 100 times more sensitive than bulk chromatin to DNase I. Although these "hypersensitive" sites may occur almost anywhere -- before genes, after genes, or within genes -- an exciting feature is that the hypersensitive sites which occur before genes appear to be related to gene activity.

Dr. Carl Wu, working first with Dr. Sarah Elgin and more recently with Dr. Walter Gilbert, mapped DNase I hypersensitive sites in the region of *Drosophila* chromatin which contains genes for heat shock proteins. The most abundant heat shock protein is named hsp 70. There are five copies of the gene for hsp 70, two at one cytogenetic locus and three at another locus. All of these genes have a site which is cleaved by the restriction endonucleases Bam and Sal. To map the DNase I hypersensitive sites, the chromatin was first treated gently with DNase I and then with one of the restriction endonucleases. After electrophoresis, the resulting DNA fragments were allowed to hybridize with a radioactively labeled piece of DNA complementary to the region containing the restriction site. The size distribution of the DNA fragments which hybridized to this probe was specific, demonstrating that DNase I cleaved only certain sites and that these sites flank and extend through the 5' end of the hsp 70 coding region (see figure below). In contrast, purified DNA which was treated in the same way was not cleaved by DNase I at preferential sites. This suggests that hypersensitivity depends upon the structure of intact chromatin.



Structure of a eukaryotic gene and flanking regions. Solid box is the gene. Open boxes are highly conserved nucleotide sequences which may be involved in transcription. Arrow shows direction of transcription.

DNase I hypersensitive sites were mapped by similar procedures for several other heat shock proteins by Drs. Wu, Elgin and coworkers. Hypersensitive sites were found at the 5' ends of all of these genes. The major hypersensitive sites were found both before and after induction of heat shock protein synthesis and also at different stages of development. Similarly, Dr. Abraham Worcel and coworkers demonstrated hypersensitive sites at the 5' ends of Drosophila histone genes in embryonic, adult, and tissue culture cells.

For some genes, however, the hypersensitive site at the 5' end may be present only in selected cell types. Dr. Weintraub and coworkers found that most of the hypersensitive sites near the genes for α- and β-globin in chicken erythrocytes are located at the 5' ends, and are absent in those genes from brain cells. Interestingly, one of the α-globin genes, which is transcribed in primitive erythroblasts, loses its DNase I hypersensitive site when its transcriptional activity ceases later in development. Drs. Wu and Gilbert compared the DNase I hypersensitivity of the rat preproinsulin II gene in chromatin from brain, kidney, liver, spleen, and a subcutaneously implanted insulin-producing tumor (insulinoma). They found that only the insulinoma had a 5' hypersensitive site.

The relationship between actual or potential transcriptional activity of a gene and the presence of a DNase I hypersensitive site may be a general phenomenon. Thus, it appears that genes which are developmentally regulated have hypersensitive sites only at particular stages of development or in certain tissues, while other genes which can be activated in any tissue always have these sites.

Two questions arise from these findings. First, what molecular mechanism underlies the hypersensitivity to DNase I? Hypersensitivity may be due to alignment of nucleosomes (repeating subunits of chromatin) along specific DNA sequences, or it may reflect the binding of certain macromolecules to these sequences, causing them to be more sensitive. The second and more interesting issue relates to the function of the hypersensitive sites. Deletion of the hypersensitive site at the 5' end of genes interferes with transcription. This suggests that these sites may be necessary for initiating gene transcription, perhaps by having a structure to which RNA polymerase or regulatory molecules can bind. However, the presence of a hypersensitive site is not sufficient for transcription, since in developmentally regulated genes (such as those for globin and insulin) the site is present prior to expression of the gene. Recent studies indicate that two highly conserved nucleic acid sequences which occur before the 5' end of many genes appear to be necessary for transcription. One sequence (TATA) is located between the hypersensitive site and the beginning of the gene. The second sequence (CCAAT) is within the hypersensitive region. Although it is not yet clear whether the CCAAT sequence contributes to DNase hypersensitivity, results from ongoing research should soon explain the relationship between structure and function of these chromatin sites.

"Covalent Modification of High Mobility Group Proteins in Chromatin May Affect DNA Transcription"
R01 GM 17383-11 (Allfrey, V.), Rockefeller University, R01 GM 27236-02 (Bhorjee, J.), University of Illinois, R01 GM 27736-02 (Levy-Wilson, B.), University of California, Irvine and R01 GM 26702-03 (Reeves, R.), Washington State University, Pullman

Chromatin is composed of a highly organized group of molecules, including DNA, histones, and non-histone proteins. In recent years, the search for chromatin-associated proteins which regulate gene expression has focused on the nonhistone high mobility group (HMG) proteins, given that name because of their behavior on electrophoretic gels. Regions of chromatin which are being actively transcribed are enriched in HMG proteins. Two of these, HMG-14 and HMG-17, are intimately associated with active chromatin, suggesting that their presence or absence may help determine whether DNA exists in a transcriptionally active or inactive form. There are, however, many copies of HMG proteins (10^5 to 10^6) in the cell nucleus, and homologous HMG proteins in a variety of species and cell types are similar. These observations have led most investigators to believe that HMG proteins are not specific enough to regulate gene transcription by themselves.

Although the lack of specificity clouds the understanding of function, new approaches are being developed. The search for possible roles for HMG proteins now involves looking for subtle differences in their structures. Recently, several NIGMS-supported investigators have discovered that HMG-14 and HMG-17 can be covalently modified after translation. Based on these results, they have speculated that these modifications could be potentially important in the regulation of gene activity.

Proteins can be modified by glycosylation, which is the post-translational addition of a sugar or a chain of sugars. Drs. Reeves, Chang, and Chung purified HMG proteins from calf thymus and mouse erythroleukemia cells and demonstrated by a variety of techniques that HMG-14 and HMG-17 contain covalently bound sugars. The discovery that HMG-14 and HMG-17 are true glycoproteins was unexpected and has interesting implications. The function of glycosidic modification of the HMG proteins is unknown, but glycosylation often serves as a mechanism by which proteins recognize each other. Preliminary experiments from Dr. Reeves' laboratory show that there may be heterogeneity among the sugars contained in different HMG proteins. Glycosylation might produce a variety of HMG proteins, each of which could interact specifically with other chromatin proteins or with particular DNA sequences.

Proteins can also be modified by acetylation and phosphorylation. These modifications are detected by incubating cells with radioactively labeled acetate or phosphate, purifying the HMG proteins, and measuring incorporation of the labeled groups into the proteins. Drs. Sterner, Vidali, and Allfrey found that HMG-14 and HMG-17 from duck erythrocytes are acetylated at the epsilon-amino position of a particular lysine residue, and Drs. Levy-Wilson and Bhorjee demonstrated that HMG-14 and HMG-17 from HeLa cells bind phosphate.

Acetylation and phosphorylation probably affect the conformation or electrical charge of proteins. Acetylation of histones, for example, neutralizes the positive charge on the portion of the molecule most likely to interact with DNA and therefore weakens histone-DNA binding. Phosphorylation causes conformational changes in the protein structure. One of the best documented examples of such an effect is the interconversion between the active phosphorylated enzyme phosphorylase a and the inactive dephosphorylated form, called phosphorylase b. Acetylation and phosphorylation might induce similar structural changes in HMG proteins and in the surrounding chromatin.

Scientists have related the acetylation and phosphorylation of HMG proteins to gene expression. One approach was to treat cells with sodium butyrate, which reversibly changes the phenotype and growth potential of certain cultured cells. Dr. Allfrey's group found that this treatment stimulated the acetylation of HMG-14 and HMG-17 in duck erythrocytes by increasing the number of acetylated molecules, by increasing the number of lysine residues that are acetylated on each molecule, and by inhibiting deacetylation. Dr. Levy-Wilson, using HeLa cells and short incubation times, did not observe acetylation of the HMG proteins. She did, however, find that sodium butyrate increased the phosphorylation of HMG-14 and HMG-17 in these cells at the same time that it promoted synthesis of many new polypeptides.

In another approach, Dr. Bhorjee attempted to relate phosphorylation of HMG-14 and HMG-17 to the position of the cells in the mitotic cycle. Following cell division (mitosis), cells enter the G_1 phase when RNA and protein synthesis occurs. Cells then enter the S phase and synthesize DNA. This is followed by the G_2 phase during which there is cell growth in preparation for the next mitosis. Cultures of HeLa cells, which were dividing synchronously, were incubated with radioactive phosphate at different times during the cell cycle.

Phosphorylation of HMG-14 was highest during G_2, while phosphorylation of HMG-17 was highest during early S. In contrast, other HMG proteins incorporated little phosphate. These results, like those observed when cells were treated with sodium butyrate, suggest that changes in gene expression are related to modifications of HMG proteins. It is significant that both acetylation and phosphorylation are reversible. This may permit rapid regulation of gene expression by the altered HMG proteins.

Many scientists believe that relaxation of chromatin structure may be required for gene activity. The experiments reported here demonstrate that HMG proteins can be modified and have the potential to alter the conformation of chromatin. So far there is no direct evidence that any of the modifications are associated with a relaxation of chromatin structure or with activation of particular genes. Furthermore, histones are also subject to modification by phosphorylation and acetylation. However, these reports provide circumstantial evidence for new and exciting mechanisms by which HMG proteins may regulate gene activity.

"Studies of DNA and the Nuclear Matrix Provide Clues to the Origins of Life"
R01 GM 23922-05 (Berezney, R.), State University of New York, Buffalo

Much recent research has focused on the structure of the nuclear matrix and its relationship to chromosome organization in eukaryotic cells. The nuclear matrix contains proteins and small amounts of RNA, DNA and phospholipids. Its structure depends on non-covalent bonds for continuity, and DNA seems to interact with a specific protein embedded in the matrix. The nature of this association remains ill-defined, although a variety of techniques have suggested that the DNA-matrix interaction is important for chromosome organization, transcription, and replication. While reports in the scientific literature are not in complete agreement about many features of DNA-matrix interactions, a number of exciting hypotheses are now being generated and tested. Among the scientists studying these issues is Dr. Ronald Berezney, whose special concern is the relationship of the nuclear matrix to DNA replication.

In recent years, Dr. Berezney and others have made basic observations which are quite intriguing. Several lines of evidence indicate that eukaryotic DNA is arranged in loops which are tightly supercoiled and fixed at each end on the nuclear matrix. The domains of supercoiling and the size of the loops are constant throughout the cell cycle. About 1-2 percent of the total DNA is actually bound to the matrix, and the attachment sites consist of 1-2 kilobases of DNA. The loops between the attachments are from 60 to 200 kilobases long, depending on the cell type.

Several investigators have suggested that the points of DNA attachment to the nuclear matrix might be related to DNA replication. Investigation of this idea has proved difficult because isolation and analysis of matrix-bound DNA is cumbersome and usually results in considerable degradation of the DNA. This means that the short, intermediate fragments of DNA which are the signalposts of replication are virtually indistinguishable from nonreplicating DNA. Dr. Berezney and his colleagues have developed a technique for isolating nuclear matrices with only moderate DNA degradation. Thus, they can detect replicative intermediates, and by using radioactive tracers, can determine if they are associated with DNA-matrix attachment sites.

Using this technique, Dr. Berezney has found that both origins of DNA replication and replicative intermediates are closely associated with the nuclear matrix. These findings suggest that DNA replication occurs along matrix-attached DNA loops. It is possible that enzyme complexes bound to the matrix promote replication and processing of the DNA by pulling the DNA loops through the complex.

Rough calculations suggest that there are about 125,000 DNA attachment sites in rat liver cell nuclei and that about 0.1 percent of the total matrix proteins may be directly involved in DNA binding. These results raise the possibility that the DNA attached to the matrix contains specific nucleotide sequences. Dr. Berezney has examined the DNA which is tightly bound to the matrix and has found no evidence for specific matrix-attachment sequences. The DNA at the attachment sites is not enriched for either repetitive or unique sequences and is virtually identical to total DNA in complexity. Thus, the DNA at attachment sites seems to be random in nucleotide sequence.

This work raises many interesting questions which Dr. Berezney and other scientists plan to pursue. Determining the structure, location, and function of replication origins promises to be a fruitful line of research. The concept of chromosomes as 3-dimensional structures, rather than as strands, will help clarify how chromosomes are organized in eukaryotes. Such studies may provide essential information to researchers who are developing in vitro eukaryotic replication systems as well as to those studying gene expression in cell-free systems. The location and mode of action of replication complexes in eukaryotic cells is an area of active research. In all of these studies, Dr. Berezney's method of isolating DNA-rich matrices will prove to be useful. Further, his results regarding the organization of DNA and the nuclear matrix should be important in understanding how chromosomes replicate.

"How do Regulatory Proteins Find Their DNA Targets?"
R01 GM 15792-15 and R01 GM 29158-01 (von Hippel, P.), University of Oregon, Eugene

Site specific genome-regulatory proteins, such as the polymerases, repressors, and activators, which are involved in transcription, initiate their action by binding to specific DNA sequences (operators, promoters, and the like). Before specific binding can occur, however, the proteins must locate and recognize these targets. The magnitude of this game of hide and seek becomes clear when one considers the E. coli cell. The E. coli genome with 10^7 base pairs in its DNA usually contains a single operator site and promoter site for each operon. The binding of a protein to its receptor site on the DNA is tight and specific for that site. However, there are 10^7 potential, nonspecific binding sites which compete with the correct binding site for the protein. Paradoxically, in the E. coli lac repressor-operator system, the observed rate at which the correct protein (repressor) and binding site (operator) come together is 1000 times faster than expected from simple collision theory. This difference between expected and observed rates has been the subject of a series of studies from Dr. Peter von Hippel's laboratory.

To explain the difference between observed and expected rates, a two-step process must be involved. The first step is the binding of a repressor protein to non-specific sites on the DNA. This is followed by a facilitated transfer of the repressor to the specific operator site. To accommodate the facilitated transfer, one of two mechanisms occurs. The first would be the random sliding of the protein along the DNA molecule until it reaches its specific binding site. The second mechanism would be the rapid and direct transfer of repressor from one part of the DNA to another by DNA intersegment transfer. To illustrate this, the repressor protein might be thought of as being bound to one arm of an octopus (DNA) with a need to reach the mouth (the specific binding site). In the first case, the molecule would roll down the suction cups to the mouth. In the other, the arm would swing around to the mouth and transfer the molecule directly.

In order to distinguish between these two mechanisms, Dr. von Hippel made use of the fact that the positions of the repressor molecules should be different for the two models. Based on theory, it was clear that the two mechanisms could be distinguished by the relationships of rate and binding constants of the repressors to DNA length and to salt concentration. The next step was to determine both equilibrium and rate constants for repressor-operator binding as a function of DNA length over a range of sodium chloride concentrations. The data were found to be consistent with a sliding mechanism. The next step will be to determine if other protein molecules which interact with specific sites on DNA, such as other repressors, polymerases, or restriction enzymes, bind via the same mechanism. More importantly, scientists must determine if this mechanism operates not only in the test tube but also in the living cell.

"Putting Together the Nucleotides: A Model in the Absence of Polymerase"

R01 GM 13435-15 (Orgel, S.), Salk Institute for Biological Studies, La Jolla, California

The central reaction responsible for the transmission of genetic information from one generation to the next is the replication of DNA. A very closely related reaction, the transcription of messenger RNA from the DNA template, is a key step in protein synthesis. In all living things, these two processes are catalyzed by complex enzymes, DNA polymerases and RNA polymerases. However, when one tries to imagine how the genetic system began, one is faced with a "chicken and egg" situation. It is impossible to produce the enzymes needed to replicate DNA or RNA without pre-existing DNA or RNA molecules. On the other hand, DNA or RNA molecules could not replicate without the help of pre-existing enzymes. Dr. Orgel believes that the solution to this paradox lies in the ability of certain RNA-like molecules to replicate either without organic catalysts or with the help of catalysts much simpler than modern enzymes. His research program is aimed at demonstrating this property of nucleic acids.

DNA and RNA are formed by stringing together four nucleotide bases designated A, U (or T), G, and C to form long chains. Replication of the molecules depends on base pairing. A double-stranded structure is formed only when an A in one strand is opposite a U (or a T) in the other, or when a G in one strand is opposite a C in the other. It is possible to unzip a double-helix into two single strands and use each separated strand as a template to synthesize a complementary strand.

Dr. Orgel's program is based on an old observation that certain long preformed strands such as UUUU... or CCCC... will attract single nucleotides, even when those are not yet joined together. Thus the alignment of single nucleotide bases, A's for example, opposite a suitable chain, UUUU..., might put the single A's in just the right position to be bonded.

$$\begin{array}{ccc} \text{U-U-U-U-U-U} & & \text{U-U-U-U-U-U} \\ & \longrightarrow & \\ \text{A A A A A A} & & \text{A-A-A-A-A-A} \end{array}$$

As part of a research program that has gone on for several years, Dr. Orgel has shown that this is indeed the case. A string of U's helps to zip together A's and only A's; a string of C's helps to join G's and only G's. However, these nonenzymatic reactions are not very efficient, and it had been impossible to join more than six to eight A's or G's. Naturally occurring nucleic acid chains are much longer.

Recently Dr. Orgel has discovered that if a string of C's is used to join together G's and a small amount of zinc is added to the reaction mixture, the reaction becomes much more efficient. In fact, as many as 40 G's can be polymerized. Furthermore, the reaction is very accurate in the sense that even if A's, U's and C's are present in the reaction mixture they are not incorporated into the long chains. This reaction, therefore, is similar to the reaction performed by an RNA polymerase. The most intriguing aspect of this discovery relates to the role of zinc. So far, only zinc has been found to work in joining G's together in the right way. Polymerase enzymes all contain a single divalent cation, zinc. It is tempting to believe that this is not a coincidence. Zinc may have been "chosen" for polymerase because it facilitates the correct joining together of bases, and the zinc-containing enzymes may have evolved from a much simpler zinc-containing molecule.

Dr. Orgel's long-term goal is to develop a more general system that works for any preformed string of bases containing U, A and G in addition to C. If he does so, he will provide a key piece of information on the way in which DNA and RNA replication could have begun on the primitive Earth. While Dr. Orgel's motivation may be the solution of a rather abstract problem, his findings are likely to have important general consequences. The DNA and RNA polymerases are among the most studied enzymes, yet little is known about how they achieve their very high efficiency and accuracy. The development of a model system which imitates the polymerases, even if it is much less accurate and efficient, will provide important basic information concerning the mechanism of action and the evolution of these important enzymes.

Genome Structure: Its Application to Genetic Function

"NIGMS Puts Genes on the Map: Recent Advances in Gene Mapping"

R01 GM 20927-07 (Davidson, N.), California Institute of Technology, and
P50 GM 20124-09 (Ward, D. C.), Yale University

For over 70 years, geneticists have been employing a variety of elegant methods to assign genes to particular chromosomes and to map genes on specific regions of the chromosome. The earliest methods were applicable only in specialized cases. Determining the relative positions of genes by the frequency of gene recombination between homologous chromosomes, for example, is useful in *Drosophila*, where crosses are easily made between flies with readily visible markers. Similarly, assignment of a gene to a particular region of a chromoosome based on microscopically observable chromosomal abnormalities is of value when a relatively large area of the chromosome is affected.

A more recent and more widely applicable procedure for gene mapping makes use of somatic cell hybridization, in which cells from two species are fused to form one cell. In the process, some chromosomes from the original cells are lost, and each clone that develops from a hybrid retains a set of chromosomes characteristic of that clone. When hybrids are made between human and mouse cells, for example, it is possible to assign a human gene to a particular chromosome by correlating its expression with the presence or absence of that particular human chromosome in different hybrid clones. Dr. Frank Ruddle (GM 09966-20) has refined this technique by transferring whole chromosomes, chromosome fragments, or purified DNA of one species into cells of another species. This has made it possible to map hundreds of human genes.

Of all the gene mapping procedures, however, the most direct and the one with the greatest potential, is in situ hybridization. With this method it should be possible to map any DNA sequence, whether it codes for an identifiable gene product or not.

Fragments of DNA are cloned in bacterial cells and radioactively labeled by growing the bacteria in the presence of ^{3}H- or ^{125}I- labeled nucleotides. The cloned labeled sequence is then reisolated for use as a probe. Chromosomes are spread on a glass slide and allowed to react with the labeled DNA fragment. This probe will hybridize, or bind, to that region of the chromosome which contains a sequence complementary to it. The unbound DNA is then washed off, and the location of the radioactivity which remains bound to the chromosomes is determined by autoradiography. In this procedure, disintegration of the radioactive isotope exposes the overlying photographic emulsion, producing small spots at the position of the hybridized sequence. In situ hybridization has been most successfully used to map DNA sequences in Drosophila polytene chromosomes which contain thousands of strands of DNA in precise alignment. Furthermore, in situ hybridization may be extended to mapping sequences in metaphase chromosomes by increasing the sensitivity of detecting the hybridized DNA.

In theory, one way to increase the sensitivity is to increase the specific activity of the radioactive label. At higher specific activities, however, the natural decay of radioactive isotopes leads to more rapid destruction of the probe and an increase in the background of the autoradiogram.

To overcome this problem, NIGMS grantees in two laboratories have recently developed novel modifications of the in situ hybridization technique. Drs. Madeline Wu and Norman Davidson labeled cloned DNA fragments with gold spheres to determine the position of the hybridized DNA. In this method, gold spheres of a uniform diameter (25 nanometers) are coated with the protein bovine serum albumin, and single-stranded bacterial DNA is covalently linked to the albumin on the spheres by a photochemical reaction. The ends of the DNA are then modified enzymatically by adding long "tails" of thymidine residues (poly (dT) tails). Cloned fragments of Drosophila DNA are modified by adding tails of adenine residues. These poly (dA) tails are complementary to the poly (dT) tails which are attached to the gold spheres.

Drosophila polytene chromosomes are spread on glass slides to form the first layer of a "sandwich". The poly (dA)-tagged DNA probe (second layer) is allowed to hybridize with the chromosomes. The modified gold spheres are then added to form the top layer of the "sandwich". Because the poly (dT) tails on the spheres pair with the poly (dA) tails on the hybridized DNA fragments, the spheres specifically mark the location where the DNA probe hybridizes. By electron microscopy, the exact chromosomal site of binding can be detected.

This procedure was tested by mapping three cloned DNA fragments whose chromosomal positions were known from conventional in situ hybridization. A probe for 5S ribosomal RNA genes mapped correctly to specific chromosomal puffs. DNA fragments lacking poly (dA) tails were not detected, and background of poly (dT)-tailed gold spheres was low. Two single-copy genes which are separated by 263 kilobases also mapped accurately in distinct bands which could be resolved from each other.

A second, very promising modification of in situ hybridization has been reported by Pennina Langer and David Ward. Initially, Dr. Ward's laboratory synthesized biotinyl-deoxyuridine 5'-triphosphate (BdUTP), a derivative of the nucleotide deoxyuridine 5'- triphosphate to which biotin (vitamin H) is attached. BdUTP substitutes for thymidine triphosphate as a substrate for DNA polymerase and thereby becomes incorporated into DNA. Bacteria containing cloned fragments of DNA are allowed to replicate in the presence of BdUTP, and this biotinyl-DNA is used as a probe for in situ hybridization with Drosophila polytene chromosomes. Its binding to chromosomes is detected by incubating the chromosomes containing the hybridized probe with a rabbit antibody specific for biotin. This reaction is followed by incubation with fluorescently tagged goat anti-rabbit antibody to form a four-layered "sandwich". Fluorescence microscopy is used to detect indirectly the hybridized DNA probe. This method, like that of Drs. Wu and Davidson, circumvents the problems caused by radioactively labeled probes, is very sensitive, and has low background. In addition, the procedure is rapid. Dr. Ward predicts that with further refinements this immunological method will be able to detect single-copy sequences on mammalian metaphase chromosomes.

The ability to map single-copy genes with precision is of great importance in understanding regulation of gene activity, modifications in the structure of the genome during development and evolution, the role of transposable DNA sequences, the mechanisms of action of proviral sequences which enter the eukaryotic genome, and the effects of gene position on expression. It will also improve prospects for genetic screening and prenatal diagnosis of genetic defects. The contributions to gene mapping made by the laboratories of Dr. Davidson and Dr. Ward give promise of adding considerably to our knowledge of gene structure and function.

"Make a Better Mouse . . . Introduction of Foreign Genes into Mouse Embryos"
R01 GM 09966-20 (Ruddle, F.), Yale University

One outgrowth of gene cloning technology has been the development of procedures which allow the introduction of foreign genes into cultured mammalian cells. In the past five years, the genes for thymidine kinase, adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase, metallothionein, and β-globin have been introduced into cells. This approach provides a powerful means of studying expression of specific genes under conditions which can be experimentally manipulated.

Cultured mammalian cells, however, are not capable of developing into an organism and, therefore, cannot be used to answer questions about the fate of genes in different tissues and at different developmental stages. To address these questions, several laboratories, including that of Dr. Frank Ruddle, have introduced genes into early mouse embryos. Dr. Ruddle's group removed fertilized eggs from mouse oviducts, microinjected DNA into the egg cytoplasm near the pronucleus, and reimplanted the eggs in foster mothers which had been made "pseudopregnant" by mating them with sterile males. The DNA used for injection into the eggs was from a recombinant bacterial plasmid (designated pST6) which contained a fragment of SV40 viral DNA which provides an origin for DNA replication. The plasmid also contained the cloned thymidine kinase gene from herpes simplex virus. Since this thymidine kinase gene is distinguishable from the endogenous mouse thymidine kinase gene, incorporation of the injected DNA into mouse cells can be detected by its ability to hybridize to a probe derived from the plasmid.

Several hundred eggs were each injected with 1000 copies of the DNA, and reimplanted into foster mothers. In due course, 78 offspring were born, all of which were normal in appearance. DNA isolated from these newborns was digested with restriction enzymes which cleave DNA at specific sequences. The resulting DNA fragments were separated by electrophoresis and allowed to hybridize with plasmid DNA. DNA from two of the newborn mice hybridized with plasmid DNA to a degree which was consistent with the interpretation that approximately one copy of the foreign DNA was present in most or all of the cells. This could occur only if the foreign genes were taken up by the eggs and were transmitted to daughter cells at each cell division.

In the two positive mice some of the foreign DNA was present as free circular molecules. However, foreign sequences were also integrated into the mouse chromosomes. The integration sites differed in the two mice, and in both cases there was extensive rearrangement of the integrated sequences. In one mouse, bacterial DNA sequences, but not SV40 or herpes virus thymidine kinase sequences, were detected. In the second, SV40 and herpes virus thymidine kinase were detected, but the thymidine kinase gene was rearranged.

In a more recent series of experiments, Dr. Ruddle's group examined in greater detail the integration of foreign genes into the host genome. By several criteria, DNA from the plasmid pST6 became integrated into the mouse DNA. Another plasmid, pIF, constructed to contain the gene for human leukocyte interferon, also appeared to be integrated. The ultimate test for integration of a gene into the mouse DNA, however, is its ability to be transmitted in a Mendelian distribution to offspring. A female mouse which was positive for pIF DNA sequences was crossed with an uninjected male. About half of the progeny from three litters inherited pIF-derived sequences, which suggests Mendelian inheritance. When the DNA of the positive offspring and of the mother were digested with restriction enzymes, and the genomic organization of the integrated human DNA compared, the resulting electrophoretic patterns were identical. A second generation also showed an identical restriction pattern, providing proof that the injected DNA was stably integrated.

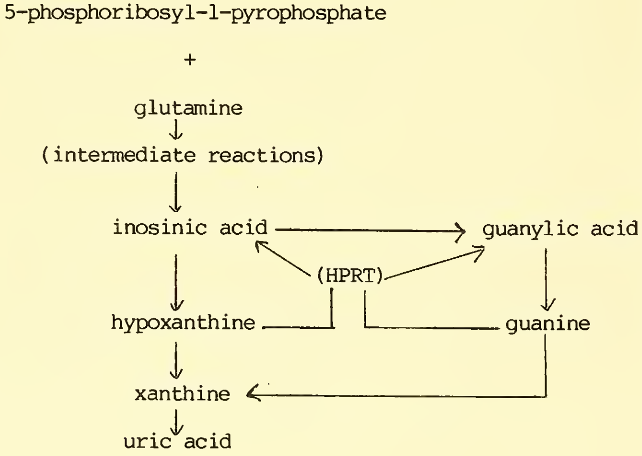
Other laboratories have been performing analogous experiments. By procedures similar to those developed in Dr. Ruddle's laboratory, cloned rabbit β-globin, human β-globin, herpes virus thymidine kinase and metallothionein genes have been introduced into mice. These genes were transmitted to some of the progeny, and in certain cases the products of the foreign genes could be detected in these animals.

The ability to introduce foreign genes into the germ line of animals is significant for several reasons. Most important, breeding mice which develop from injected eggs could provide large numbers of identical animals for use in studying the control of gene expression. These procedures will also permit the study of crossover events within a well-defined genetic region, facilitate gene mapping, and help explain how sequences surrounding the site of integration affect gene expression. In addition, it may be possible to use offspring of injected animals to produce large amounts of developmentally regulated gene products. While introduction of foreign genes may not make a better mouse, this technology will provide a valuable new tool for developmental geneticists.

"Improved Understanding and Potential New Treatment of Lesch-Nyhan Syndrome"
R01 GM 28223-02 (Friedmann, T.), University of California, San Diego

Dr. Theodore Friedmann and his colleagues have become the first researchers to clone the gene which causes Lesch-Nyhan syndrome, a severe, X-linked enzyme disorder. Scientists from other laboratories have previously cloned similar mammalian genes, but Dr. Friedmann is the first to isolate the human gene. Because of the importance of this gene to human health, its isolation and characterization are especially significant. Some speculate that this work may eventually lead to gene therapy of Lesch-Nyhan syndrome and gouty arthritis.

The gene which Dr. Friedmann has cloned codes for the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT), an enzyme which is needed to recycle nucleotide bases for DNA and RNA synthesis, as shown below:



Defects in the HPRT gene have been linked to two important human diseases -- gouty arthritis and Lesch-Nyhan syndrome. The latter disorder is a severe neurological syndrome, characterized by psychomotor retardation, hypouricemia, and bizarre behavior. Patients with Lesch-Nyhan syndrome compulsively chew their hands and lips, especially when they are upset. Because the HPRT gene is on the X-chromosome, it has become an important model for studies of X-linked inheritance. The gene has an additional important feature which facilitates its use in genetic studies. Cultured cells deficient in the HPRT gene ($HPRT^-$) can be easily isolated from normal cells ($HPRT^+$) by growing the cells in special medium. HPRT+ cells grow only in HAT medium, which contains hypoxanthine, aminopterin, and thymidine. $HPRT^-$ cells can be isolated from medium containing azaguanine. Thus, assays for the presence or absence of the gene are fairly easy and reliable.

The HPRT enzyme is a multimer with a subunit size of about 26,000 daltons. The HPRT enzyme isolated from Lesch-Nyhan patients has abnormal biochemical properties, which suggests that it is structurally altered. However, some human $HPRT^-$ cells, when fused with mouse cells, express normal human HPRT activity. This suggests that regulatory elements may be involved in some cases. Thus, Lesch-Nyhan syndrome may be a family of disorders related to several structural or regulatory mutants of the HPRT gene.

Dr. Friedmann used a variety of well established recombinant DNA techniques to isolate the HPRT gene. DNA from normal cells was purified, and the whole DNA was cut into fragments with several restriction enzymes. The fragments were sorted according to size, and each group was tested for the presence of the HPRT gene. Fragments showing HPRT activity were then used to construct plasmids. The plasmids could be amplified many times to produce sufficient DNA and mRNA to carry out detailed studies of the gene. Dr. Friedmann then showed that the cloned HPRT DNA became incorporated into chromosomes of the cells, thereby transforming $HPRT^-$ cells into $HPRT^+$ cells.

Dr. Friedmann's observations may open the door to studies of gene therapy of Lesch-Nyhan individuals. If the cloned gene can transform HPRT lymphoblast cells, and if some of these cells can be transferred successfully to patients with Lesch-Nyhan syndrome or gouty arthritis, successful treatment for these disorders may result. Dr. Friedmann's work is the first important step down a long and exciting road.

Genetics Program

Program Organization and Professional Staff

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David P. Beck, Ph.D.	Deputy Director

Molecular and Medical Genetics Section:

David P. Beck, Ph.D.	Chief
James Cassatt, Ph.D.	Program Administrator
Irene Eckstrand, Ph.D.	Program Administrator
Barbara Williams, Ph.D.	Program Administrator
George Woolley, Ph.D.	Program Administrator

Genetics of Growth and Differentiation Section:

Carl Rhodes, Ph.D.	Acting Chief
Fred Bergmann, Ph.D.	Program Administrator
Judith Greenberg, Ph.D.	Program Administrator
Dorothea Miller, Ph.D.	Program Administrator

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Dora Sullivan	Grants Clerk
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PHARMACOLOGICAL SCIENCES PROGRAM

SCOPE

This report reviews the activities of the Pharmacological Sciences Program for Fiscal Year 1982.

OBJECTIVES

The Pharmacological Sciences Program aims to foster and support research which provides an improved understanding of the biological phenomena and related chemical and molecular processes involved in the actions of therapeutic drugs. In addition, the research supported by the program has the potential to contribute to a delineation of basic biological mechanisms using drugs as tools and probes.

RESEARCH

The research efforts supported by the Pharmacological Sciences Program are divided into two major areas. The biorelated chemical processes portion of the program supports basic investigations directed first, at understanding biological mechanisms at the chemical or molecular level and, second, at the development of new chemical methodology aimed at the synthesis of complex molecules of biological interest. The pharmacological research portion of the program supports investigations into the nature of the interactions between small molecules and receptors, pharmacological modulation of basic physiological processes at the molecular and systems levels, and studies of these interactions in the intact organism, including human (clinical) pharmacology and pharmacokinetics.

The Pharmacology Research Associate Program (PRAT) is a unique endeavor of the Pharmacological Sciences Program. Under its auspices, eleven young scientists are selected for a two-year period of postdoctoral research under the tutelage of a National Institutes of Health (NIH) or Alcohol, Drug Abuse, and Mental Health Administration (ADAMHA) intramural scientist. During Fiscal Year 1982, the 22 first and second year PRAT fellows were working in the laboratories of seven of the institutes. Of the current fellows, eight are M.D.'s, including four with the dual M.D./Ph.D. degrees.

Dr. Lee E. Eiden, who obtained his Ph.D. in medicinal chemistry from the University of Kansas, has been working in the Laboratory of Clinical Science, National Institute of Mental Health (NIMH) under the preceptorship of Dr. Michael Brownstein. As part of his interest in the distribution and function of vasoactive intestinal peptide (VIP) in the brain, Dr. Eiden and his colleagues have undertaken a systematic quantitation by radioimmunoassay of this substance in specific nuclei of the brain stem. They found that, in general, the highest concentrations of VIP were in the dorsal regions and that its distribution was not similar to that of other neuropeptides and biogenic amines. Their data suggest that VIP does not have a specific role in mediating transmission in either the motor or the sensory nuclei of the brain stem.

Regional distribution of peptidergic systems within the brain has been an interest of PRAT Fellow, Thomas O'Donohue, Ph.D., from Howard University, assigned to the Laboratory of Clinical Science, NIMH, with Dr. David Jacobowitz. Dr. O'Donohue has chosen to study N-acetylation as a regulatory mechanism in opiomelanotropinergic cells and neurons. He investigated the regional distribution of an enzyme, "MAT", which is responsible for acetylating α-melanocyte stimulating hormone (αMSH) in the brain and pituitary glands of rats. Although MAT activity was found to be about 10-fold higher in the pituitary gland than in the brain, no clear positive correlation between αMSH content and total MAT was found in regions of the brain. However, Dr. O'Donohue's work has provided evidence for the presence of two distinct MAT enzymes. MAT 1 appears to be a general acetyltransferase while MAT 2 appears to be specific for αMSH and may be localized differently than the general MAT 1 activity. These investigators have also shown the existence of an endorphin acetyltransferase in the intermediate lobe of the pituitary gland. Selective enzymatic activation or inactivation of αMSH and βendorphin by acetylation would appear to provide a sensitive mechanism for acutely regulating the secretory output of a multi-hormone endocrine cell or a multineurotransmitter neuron.

Dr. Adelaide Siegl (who obtained her Ph.D. from Thomas Jefferson University) working in the Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Diabetes, and Digestive and Kidney Disases (NIADDK), with Dr. John Daly, has been investigating the role of the diterpene, forskolin, in the regulation of adenylate cyclase activity. Forskolin is isolated from a genus of plants used in ethnomedicine to treat heart disease, spasmodic pain, painful micturition, and convulsions. Dr. Siegl's work has shown that micromolar concentrations of forskolin markedly inhibit human platelet aggregation and also dramatically augment the ability of prostaglandins PGI_2, PGD_2, and PGE_1 to inhibit platelet aggregation. Forskolin is a potent activator of cyclic AMP generating systems in platelets, and the mechanism of the forskolin-induced reduction in platelet aggregation appears to be via the cyclic AMP system. The prostaglandins, in turn, cause a four to five fold increase in the effect of forskolin in activating adenylate cyclase in these cells. Because of its ability to potentiate receptor-mediated activation of adenylate cyclase, forskolin may provide a tool for selectively manipulating specific cyclase systems in intact organisms.

RESEARCH TRAINING

The Pharmacological Sciences Program has supported research training in clinical pharmacology and the pharmacological sciences under the authority of the National Research Service Act since 1975. Training is supported by both institutional and individual fellowships. In Fiscal Year 1982, institutional awards at 34 institutions in 23 states provided support for 364 predoctoral fellows to receive broad-based, multidisciplinary training in the pharmacological sciences. While generally based within departments of pharmacology, these programs involve integrating aspects of biochemistry, medicinal chemistry, physiology, neurobiology, immunology and other related areas into training for solving problems of pharmacological interest.

Postdoctoral training programs in clinical pharmacology were supported at seven institutions in five states. Eleven fellows were trained by these programs during Fiscal Year 1982. In addition, two fellows chose to pursue the clinical pharmacology track of the Pharmacology Research Associate Program.

Individual postdoctoral fellowships are awarded competitively to young scientists in the areas of clinical pharmacology, biorelated chemistry and pharmacology. In Fiscal Year 1982, 43 such awards were made, one of which was in clinical pharmacology.

The PRAT Program is the only intramural activity of the National Institute of General Medical Sciences. It provides opportunities for 22 outstanding postdoctoral fellows to spend two years in one of the intramural laboratories of the NIH. There are currently 60 preceptors who participate in the program, but a fellow entering with a background in the pharmacological sciences may choose to work in any laboratory of the NIH. Currently fellows are pursuing such diverse research problems as defining the mechanisms of signal transduction through biological membranes, the clinical pharmacology of some cancer chemotherapeutic agents, or genetic regulation of drug metabolism. In addition, a clinical pharmacology option under the guidance of Dr. William Z. Potter is provided for M.D.s who wish to acquire training in the application of pharmacological research principles to clinical research. Two fellows are participating in this aspect of the PRAT program.

Research Career Development Awards are considered by this program only from those investigators who have expressed a commitment to research careers in clinical pharmacology. It is the intent of both the National Institute of General Medical Sciences and the Pharmacological Sciences Program that this mechanism will enable such individuals to develop into outstanding clinical investigators who will be able to apply principles of basic pharmacological research to problems of clinical relevance. Two individuals are currently supported by the mechanism. No new awards were made in Fiscal Year 1982.

ORGANIZATION AND MANAGEMENT

Several staff changes occurred during Fiscal Year 1982. James Gilliam, M.D., Ph.D., joined the Program in December 1981, as a program administrator with special interests in clinical pharmacology. Warren Jones, Ph.D., program administrator, moved to the Cellular and Molecular Basis of Disease Program to assume responsibility for the administration of a grant portfolio related to enzymic mechanisms. Dr. Jones also administers some grants dealing with peptide synthesis in the Biorelated Chemistry area, Pharmacological Sciences Program.
Mrs. Mary Beth Gallagher joined the program as a grants assistant.

RESEARCH HIGHLIGHTS

"Clinical Pharmacology of Antiarrhythmic Drugs (Encainide)"

P50 GM 31304-01 (Oates, J.), Vanderbilt University Medical Center

Encainide is a relatively new, highly potent drug which serves as a prototype of a family of new antiarrhythmic agents currently undergoing clinical testing. After a period of high enthusiasm, encainide was in danger of falling into disfavor because of the difficulty clinicians experienced trying to correlate dosage and response. The drug did not appear to have the predictability of response necessary to assure safe use. The selection of a drug dosage which maximizes therapeutic effects while minimizing side-effects is particularly important for agents with low therapeutic ratios.

During the investigation of new antiarrhythmic drugs, an awareness of the relationship between concentration of the drug in plasma and the pharmacological actions observed can lead to early detection of active metabolites. This was demonstrated during an evaluation of the antiarrhythmic, electrocardiographic and dose-response effects of encainide by Dr. Raymond Woosley and his colleagues.

Patients with total abolition of ventricular ectopy and ventricular tachycardia had rapid encainide elimination ($T_{1/2}$ = 2-4 hr.), marked electrocardiographic effects at variable dosages (12.5-75 mg every 6 hr.) and highly variable (but low) plasma concentrations of encainide. In contrast, a single non-responding patient had much slower encainide elimination ($T_{1/2}$ = 7.6 hr.), higher plasma encainide concentrations and, yet, no antiarrhythmic or electrocardiographic effects at dosages up to 125 mg every six hours. Chromatographic analysis of plasma from the responding patients revealed that the O-demethyl metabolite (ODE) of encainide was present in greater quantities than parent drug; yet in the non-responder it was hardly detectable except at very high dosages of encainide. Subsequent studies in a larger number of patients found that about ten percent of patients appear to lack the ability to rapidly O-demethylate encainide, and in this group encainide accumulates to high plasma concentrations. Dr. Woosley and his co-workers have accumulated a great deal of indirect evidence indicating that the metabolites of encainide contribute to the antiarrhythmic efficacy seen during encainide therapy. To test their hypothesis that one or more of the metabolites of encainide was pharmacologically active, these investigators compared the antiarrhythmic and electrocardiographic effects of ODE and encainide in an animal model of ventricular arrhythmias, the aconitine-intoxicated rat. ODE was 50-fold more active than encainide against aconitine-induced ventricular tachycardia. Plasma analysis indicated that the metabolite was present shortly after encainide administration in concentrations that appeared to be active in this model. However, inhibition of metabolism showed a protective effect of encainide alone. Therefore, both parent compound and the O-demethyl metabolite were active, the metabolite being the more active of the two.

The correlation of plasma concentration and antiarrhythmic activity of ODE indicates the potential for a safer, more predictable use of this metabolite as compared to encainide. Final proof of the relative safety and efficacy of all the metabolites will be determined by their administration to patients with ventricular arrhythmias.

"Regulation of β-Adrenergic Receptor Function"

R01 GM 29522-01 (Perkins, J.), University of North Carolina at Chapel Hill

Dr. Perkins' group has been studying the agonist-induced down-regulation of the response of the adenylate cyclase system to catecholamines. Short-term exposure of human astrocytoma cells to catecholamines results in a reduction of the capacity of the catecholamine-occupied β-adrenergic receptor (BAR) to increase adenylate cyclase activity. While no change in the number of BAR or in any other parameters of adenylate cyclase activity was apparent, the steady-state level of adenylate cyclase activity following incubation of cells with isoproterenol dropped to a new level of 40-50 percent of control. Recovery was rapid and complete upon removal of the catecholamine from the medium. The investigators propose that an uncoupling occurs between the receptor and the adenylate cyclase such that occupation of the BAR is no longer capable of stimulating enzyme activity. Receptor binding studies also indicated that the early phase of desensitization involved uncoupling of BAR and adenylate cyclase. Following short incubations

(5-30 minutes) with isoproterenol (ISO), the apparent affinity of the agonist for inhibition of iodohydroxypindolol (IHYP) binding was reduced ten-fold with no change in the number of receptors. The time course of this change in affinity of agonist binding was similar to the time course of loss of adenylate cyclase activity. Both binding affinity and isoproterenol-stimulated adenylate cyclase activity returned to control levels with similar time courses following removal of isoproterenol.

Dr. Perkins suggests the following kinetic model for the agonist-specific desensitization:

$$BAR_N \overset{+ISO}{\rightleftharpoons} BAR_U \xrightarrow{+ISO} BAR_L$$

where BAR_N is the native receptor, BAR_U is the altered form of the receptor which binds agonist but does not activate adenylate cyclase, and BAR_L is the form of receptor which occurs after long exposure to isoproterenol and which is not detectable even by antagonist binding techniques. The initial process can be envisioned as involving the reversible formation of the altered receptor. During short-term incubation with catecholamine, the receptor exists in both the native and altered states and the steady state levels of these two forms (and subsequent response of adenylate cyclase) is determined by the balance of the forward and reverse reactions. Long term incubation with catecholamine results in a loss of receptors and conversion of the BAR to the IHYP undetectable form (BAR_L) from which recovery is very slow.

The physical properties of the BAR_U differ from those of BAR_N. While native receptors sediment as a single peak in sucrose gradients with adenylate cyclase from isoproterenol-stimulated control or desensitized cells, the receptors from desensitized cells migrate as two peaks. Receptors from the light peak exhibit properties similar to those of uncoupled receptors. The appearance of this "light" form of receptor coincides on a temporal basis with the agonist-induced uncoupling reaction. The cellular location of these receptors is speculative at this point since they sediment at densities lighter than markers for the plasma membrane. This "light" receptor peak does sediment at equilibrium densities similar to those for Golgi apparatus, lysosomes, and endoplasmic reticulum, suggesting that the uncoupled receptors might exist in cytoplasmic vesicles resulting from agonist-induced selective endocytosis of receptor protein.

"Pharmacokinetics - Pharmacodynamics of Immunoregulating Agents"

P50 GM 26691-03 (Benet, L.), University of California, San Francisco

Among the challenges of pharmacokinetics has been to relate the kinetic measures of a drug in the body to the pharmacological activity of that drug, and hence to specific measures of clinical efficacy and toxicity. In the case of immunoregulating drugs, these challenges have been amplified by the difficulties in quantitating these drugs in biological fluids at the levels present after administration of therapeutic doses, and, perhaps more important, defining precise measures of pharmacological activity which can be related to clinical efficacy.

Dr. Leslie Benet and his colleagues have been pioneering in the field of "immunopharmacokinetics" to find better ways of applying pharmacokinetics to this expanding area of therapeutically important drugs. These investigators have developed sensitive and specific assays for azathioprine, 6-mercaptopurine, and for prednisone and prednisolone as well as for the endogenous glucocorticoids. In addition, because of the difficulty of defining any quantitative measure of efficacy and toxicity in the whole patient, intermediate measures of immunological effect which can be applied to immunoregulatory agents have been developed. These workers have selected the inhibition of the mixed lymphocyte reaction (MLR) as a rational model for quantifying immunosuppressive activity in plasma.

These investigators have recently studied the effects of prednisone, prednisolone and hydrocortisone on human peripheral blood lymphocyte blastogenesis in a two-way MLR. As measured by the inhibition of the MLR, prednisolone is about twice as potent as hydrocortisone. Despite their differences in potency, both prednisolone and hydrocortisone yield the same maximal inhibition. Prednisone, on the other hand, shows no significant immunosuppressive activity despite the significant slow conversion of prednisone to prednisolone in the MLR by 11-hydroxylation. The presence of prednisone had no effect on the inhibition due to prednisolone and hydrocortisone.

Employing modifications of the MLR assay at 50 percent plasma concentration in the mixed lymphocyte cultures, a quantitative measure of immunosuppression, apparently related to pharmacokinetics, was developed. This measure, the area under the inhibition-time curve, is calculated in a manner similar to the commonly used pharmacokinetic parameter, the area under the drug-plasma concentration time curve. Dr. Benet and his group have used this new methodology to study the pharmacokinetics of prednisolone and prednisone in stable renal transplant patients and in patients with oral mucocutaneous vesiculoerosive disease. In addition, the pharmacokinetics of prednisolone was compared between cushingoid and non-cushingoid patients. While, in contrast to previous literature reports, a significant decrease in prednisolone clearance in cushingoid patients was not found, they did find a significantly higher level of hydrocortisone in cushingoid patients following exogenous glucocorticoid dosing.

Additional studies have been aimed at extending and validating the correlation between the intermediate index of immunosuppressive activity and pharmacokinetics for this important class of drugs and their metabolites.

"The Role of Prostaglandins in Hemopoiesis"

R01 GM 30410-01 (Ziboh, V.), University of California at Davis

The granulocyte-macrophage progenitor cells are committed hematopoietic cells which are capable of proliferating and differentiating in the presence of a glycoprotein termed "colony stimulating factor" (CSF). The name derives from the fact that cloning of these progenitor cells *in vitro* in the presence of CSF produces colonies of hematopoietic cells. Various small molecules, including the prostaglandins, are known to alter CSF-induced proliferation.
Dr. Vincent Ziboh has been investigating the role which prostaglandins play in the stimulation of granulopoiesis.

Using both turpentine-induced rat myeloid hyperplastic bone marrow cells and sodium caseinate-induced peritoneal monocyte/macrophages, labelled with ^{14}C-arachidonic acid, Dr. Ziboh and his colleagues were able to show that CSF challenge resulted in the release of arachidonic acid from as yet unidentified membrane phospholipid(s). The investigators speculate that the release of arachidonic acid is due to activation of a cellular phospholipase A by CSF.

The arachidonic acid which is released by CSF is subsequently transformed into products of both the cyclooxygenase (prostaglandins) and lipoxygenase (leukotrienes) pathways. The hyperplastic granulocytic marrow cells generated a significantly greater proportion of lipoxygenase products than did the monocyte/macrophage population, implying that the two cell populations may have differing functional roles in hemopoiesis.

Hydrolysis of free arachidonic acid from cellular phospholipid is the rate-limiting step in the phospholipid-arachidonic acid-prostaglandin/leukotriene cascade. The investigators also tested the hypothesis that it is the products of this cascade which produce growth stimulation. Mouse bone marrow cells were incubated in the presence and absence of various inhibitors of the lipoxygenase and cyclooxygenase pathways. Drugs which inhibited both pathways also inhibited CSF-induced proliferation of the marrow cells. A selective inhibitor of the lipoxygenase pathway (15-HETE) inhibited CSF-induced colony formation while indomethacin, which at low concentrations selectively inhibits the cyclooxygenase pathway, had no inhibitory effect on CSF-induced colony formation. This implies that it is the products of the lipoxygenase pathway, the leukotrienes, rather than the prostaglandins which mediate granulopoiesis in response to CSF. Further support for this was generated by adding a mixture of the lipoxygenase products isolated from rat peritoneal neutrophils to marrow cells incubated with a lipoxygenase-cyclooxygenase inhibitor. The inhibition of CSF-induced proliferation was reversed by the addition of these lipoxygenase products and colony formation once again occurred.

"Use of Macrophage-like Cell Lines"
R01 GM 29042-02 (Rosen, O.), Albert Einstein College of Medicine

Taxol, which possesses antileukemic and tumor inhibitory properties, is a novel diterpene isolated from the plant *Taxus brevifolia*. Dr. Susan Horwitz at Albert Einstein College of Medicine has been investigating the actions of this drug both *in vitro* and in the macrophage-like cell line, J774.1, in an attempt to identify the mechanism of cytotoxicity of this drug. Unlike other antimitotic drugs, such as colchicine or vinblastine, which inhibit microtubule formation, taxol enhances both the rate and yield of microtubule assembly. The critical concentration of microtubule protein required for assembly is reduced and the microtubules formed are stable to depolymerization by calcium or cold. Taxol also assembles tubulin under conditions in which polymerization would not normally occur, such as in the absence of microtubule-associated proteins, exogenously added GTP, organic buffer, or at low temperatures. Taxol appears to act by stabilizing the interactions between tubulin dimers that lead to microtubule polymer formation. In HeLa cells, taxol produces unusual microtubule cytoskeletons characterized by the presence of discrete microtubular bundles, and cells treated with the drug accumulate in G_2 and M phases. Under conditions which prevent the characteristic taxol-induced bundle formation in J774.1 cells, binding of taxol to intact cells is retained. Thus, binding of taxol to microtubules is not in itself sufficient to promote bundle formation.

Using radiolabeled taxol and either intact J774.1 cells or purified tubulin from brain, Dr. Horwitz has found that taxol binds in a specific and saturable manner to a single, high affinity binding site on the microtubule. The stoichiometry of this binding with the tubulin dimer concentration approaches one. Maximal binding occurs at drug concentrations which produce maximal growth inhibition. Structure-activity relationship studies with a series of natural and semisynthetic taxanes have shown that both cytotoxicity and microtubule stabilization in vitro require an intact taxane ring and ester side chain. Small changes in the side chain and in ring substituents can, however, be tolerated without loss of biological activity. Dr. Horwitz hopes that these studies will permit the incorporation of a fluorescent moiety into the drug so that it can be used as a probe for microtubule assembly processes. In addition, genetic variants of the J774.1 line that are highly resistant to the growth inhibitory properties of taxol have been isolated and cloned. These are being used to study the interaction of taxol with tubulin and its associated proteins in an effort to further understand the mechanism of action of this unique cytotoxic agent.

"Structure-Function Relationships of Lectins"
R01 GM 21882-08 (Etzler, M.), University of California, Davis

Many plants and animals contain agglutinins called lectins that can combine specifically with erythrocytes and other types of cells. The hemagglutinating properties of these lectins are due to their complementarity for specific carbohydrate residues on the cell surface. Lectins are quite abundant in the seeds of leguminous plants and differ from one another in their specificities. To date more than 800 plant species have been found to contain lectins, but only a relatively small number of these lectins have been rigorously studied.

The specificities of lectins for carbohydrate residues have made them valuable analytical tools for studies of complex carbohydrates, both in solution and on the cell surface. In fact the observations that some lectins can specifically agglutinate tumor cells have led to a large number of studies using lectins to distinguish between normal and transformed cells. In addition to these uses, some lectins have been found to have mitogenic properties, and several lectins are known to be very toxic to mammalian cells.

All plant lectins characterized to date have been found to be oligomers of peptide or glycopeptide chains. Although some lectins are composed of identical subunits, other lectins contain more than one type of subunit. Superimposed on this subunit heterogeneity is the finding that many lectins exist in multiple molecular forms, called isolectins, which may or may not have different specificities.

Dr. Marilynn Etzler and her colleagues have been studying the lectins of the seeds of Dolichos biflorus (Horse gram) for a number of years. These investigations have consisted of structural studies, comparative studies of various lectins and their modified forms, and studies of the development of the Dolichos biflorus lectin during the life cycle of the plant.

The Dolichos biflorus seed lectin is a glycoprotein. The carbohydrate unit contains mannose and N-acetylglucosamine and is linked to the protein through a N-acetylglucosaminyl-asparaginyl linkage. Structural analysis was by pronase digestion, alpha-mannosidase treatment, periodate oxidation and exhaustive methylation followed by gas-liquid chromatography and mass spectrometry. The data indicate that the glycopeptide is a branched structure with mannose at the branch point and that the branches are terminated by nonreducing alpha-mannosyl residues.

The NH_2-terminal amino acid sequences of the first thirty residues of subunits I and II were determined and found to be identical. COOH-Terminal amino acid analysis of the individual subunits showed a sequential release of leucine, serine and valine from Subunit I and aspartate, leucine and proline from Subunit II. A comparison of the peptide maps from isolated Subunits I and II showed that all peptides were common to both subunits with the exception of one. This unique peptide was shown to be derived from the COOH-terminal of the subunit. The following diagram summarizes the data to date:

Carbohydrate
SUBUNIT I / Met Ser Val Leu

--Identical Sequence--

----Identical Peptide Maps------------

SUBUNIT II Met Pro Leu Asp
/
Carbohydrate

Metal content of the lectin was investigated by atomic absorption spectrometry. The lectin contains Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}. Demetallization of the lectin inactivated it as tested by affinity electrophoresis. Full activity could be restored by the addition of Ca^{2+} alone or by a combination of the metal ions. None of the other metal ions added individually restored full activity.

The development and distribution of the lectin during the life cycle of the plant was followed using a sensitive radioimmunoassay. The lectin was first detected in the seed of the plant 27 days after flowering and rapidly attained the high level of lectin present in the mature seed. The lectin is confined to the cotyledons and decreases at about the same rate as the other protein reserves during the absorption of the cotyledons after germination of the seeds. No lectin was detected in the roots of the plant throughout its life cycle. Although the stems and leaves were initially thought to contain some lectin, these tissues were found instead to contain a glycoprotein that cross reacts with antibodies against the seed lectin.

The cross reactive material (CRM) was isolated from the stems and leaves and found to have an amino acid and carbohydrate composition similar to that of the seed lectin. The CRM is a dimer with one subunit identical in electrophoretic mobility to Subunit I of the seed lectin and a second unit of higher molecular weight. Both subunits have the same NH_2-terminal sequence. The available data suggest that both the CRM and the seed lectin subunits may represent different degrees of completion or modification of a common polypeptide chain.

"Norpace® Kinetics and Bioavailability in Heart Failure"

R01 GM 28424-02 (Lima, J.), Ohio State University

The degree of drug binding to plasma proteins is an important determinant not only of the drug's metabolism and distribution, but also of its pharmacodynamic activity because only free, unbound drug in plasma is available to interact at the cellular level to produce a response. Moreover, the clearance of most drugs from blood is restricted to their free, unbound fractions. The fraction of drug bound to plasma proteins is inversely related to the total (free + bound) concentration of the drug in plasma over a wide concentration range. This relationship between total plasma concentration and the fraction of the drug bound to plasma protein is not clinically important for most drugs because the bound fraction is usually constant over the relatively narrow plasma concentration range achieved following administration of therapeutic doses to patients. It has been thought for many years that albumin is the major protein which binds drugs in human plasma. More recently, however, it has been recognized that there is another component of human plasma which is also responsible for a significant amount of drug binding, particularly in the case of basic drugs. This component is α_1-acid glycoprotein (AAG).

Disopyramide phosphate (Norpace®), a relatively new antiarrhythmic drug, is a basic drug which is moderately to highly bound to plasma protein. It is an unusual compound in that its bound fraction appears to be concentration-dependent at therapeutic plasma concentrations. Consequently, its free fraction is also concentration-dependent in the therapeutic dose range. In addition to its apparent dependence on concentration, the bound fraction of disopyramide appears to vary according to the source of protein used in binding studies _in vitro_. Widely differing extents of binding of disopyramide to plasma protein have been reported.

Dr. John Lima has been trying to explain some of these anomalies in disopyramide distribution by comparing the binding of disopyramide to protein in various sources commonly used in protein binding studies, and by studying the influence of concentration-dependent protein binding on the pharmacokinetics and pharmacodynamics of the drug in human subjects. Previous work from Dr. Lima's laboratory had indicated that as much as 35 percent of disopyramide was bound to two independent sites on albumin. More recent work indicates that these two sites are on different proteins. Equilibrium dialysis binding studies of disopyramide to proteins in human plasma and serum have now been used to measure binding constants for these sites. The first binding site has a higher affinity (approximately 800-fold) and lower capacity for disopyramide as compared to the second site. The second site accounts for only about 5-10 percent of the binding of the drug at concentrations ranging from 10^{-7} - 10^{-3}M and is identical to the binding of the drug to albumin.

The similarity in the binding constants between AAG and the first class of binding sites suggests that these are identical as well. Thus most of the binding of disopyramide to human plasma protein is to AAG. This binding is temperature independent in the temperature range of 25° to 37° and is not affected by freezing, storage, and thawing of the plasma, indicating an ionic-type binding.

Dr. Lima's group has measured disopyramide binding to plasma from normal volunteers, to donor plasma obtained from a blood bank and to plasma and serum from patients in the cardiac intensive care unit. The affinity constants characterizing the high (AAG) affinity binding sites were similar for cardiac patient and donor plasmas but both were lower than for normal plasma. There was no difference noted for the low affinity site. The capacity constant, a measure of how much drug is bound, for cardiac patient serum was nearly twice that for normal volunteer serum. Despite similar affinity constants for cardiac patient serum and for blood bank donor serum, the capacity constant for cardiac patient serum was much higher than for donor serum. In an attempt to explain this anomalous relationship, the investigators used dialysis and absorption techniques to restore binding in the donor serum. This led to the theory that there exists in donor serum a competitive inhibitor for disopyramide binding. The binding of several basic drugs to serum and plasma proteins has been found to be reduced following contact of blood with the rubber stoppers of blood collection tubes. Work by Lima's group and others now indicates that plasticizers leak out of the storage bags used by blood banks and these plasticizers compete with basic drugs like disopyramide for the high affinity binding site of AAG. In addition, Shand and co-workers have shown that drug binding to protein can be altered by competition with heparin when indwelling heparinized catheters are used to obtain samples.

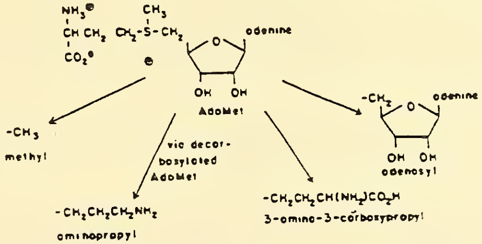
The investigators suggest that the differences in the affinity constants for cardiac patient and normal sera are probably due to competition for AAG binding by other basic drugs which the patient may have been receiving for his condition (e.g., lidocaine). The difference in the capacity constants is most likely a result of disease-induced increases of AAG. A number of disease states (e.g., myocardial infarction) as well as stress, trauma and surgery are known to stimulate the production of AAG.

These findings may be clinically significant since plasma monitoring of drugs is used as a more sensitive indicator of predictive response than administered dose. Often what is measured is total plasma concentration (free + bound). In the case of disopyramide, this total measure does not correlate with at least one pharmacodynamic response, prolongation of the isovolumic contraction period. This response is a function of the serum concentration of free disopyramide. In addition, attempts to measure free or bound fractions of drug are complicated by several artifacts which can give erroneous estimates of the amount of drug bound and by the fact that the drug binds to two serum proteins. All of these factors need to be taken into account before therapeutic drug monitoring can be used as a guide in adjusting the dose of disopyramide in cardiac patients.

"Structural Analogs of S-Adenosylmethionine"

R01 GM 29332-09 (Borchardt, R.), University of Kansas

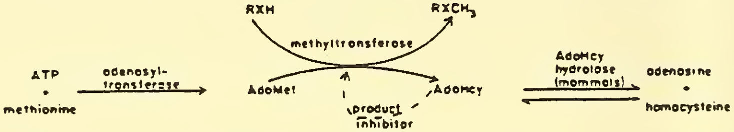
Since S-adenosylmethionine (AdoMet) was discovered in 1952 by Cantoni, it has been shown to be one of the more versatile molecules found in biological systems. As the figure below shows, AdoMet is capable of serving as a donor of four different



moieties, including the aminopropyl group, which is utilized in the biosynthesis of spermine and spermidine, and the adenosyl group, which can be added or removed from the enzyme pyruvate-formate lyase as a regulator of the enzyme's activity. AdoMet has been most widely studied as a methyl donor for numerous cellular transmethylations. The enzymes which mediate these reactions, the AdoMet-dependent methyltransferases, have been shown to play an important role in the biosynthesis and degradation of small molecules (e.g., dopamine, epinephrine, and histamine) and in the modulation of the properties of macromolecules (nucleic acids, proteins, and lipids). Many of the small molecule methyltransferases have well-defined roles in the cell. Examples include: histamine N-methyltransferase which catalyzes a methyl group transfer to histamine as part of the major catabolic pathway for histamine in mammals; catechol-O-methyltransferase which catalyzes the methylation of both endogenous catecholamines such as dopamine and epinephrine and certain exogenous materials such as the drugs isoproterenol and L-dopa.

Until recently, it was more difficult to define the exact physiological functions of many of the macromolecular methylations. However, it is now apparent that AdoMet-dependent methylations are one of the general mechanisms for regulating the activity of macromolecules in both procaryotes and eucaryotes. This modification of macromolecules by methylation has been shown to play an integral role in processes such as protein synthesis (mRNA and tRNA methylations), DNA replication (DNA methylation), gene regulation (DNA methylation), bacterial chemotaxis (protein esterification), leucocyte chemotaxis (protein esterification and phospholipid methylation), and neurosecretory processes (protein esterification and phospholipid methylations).

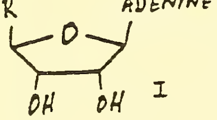
Because of their importance in controlling numerous cellular processes, the AdoMet-dependent methyltransferases have attracted considerable attention. Among a number of NIGMS-supported investigators studying these enzymes is Dr. Ronald Borchardt, who has been developing inhibitors of the AdoMet-dependent methyltransferases. The rationale used by Dr. Borchardt in the development of these inhibitors is implicit in the general reaction scheme for AdoMet-dependent methylations that is shown in the figure below. This scheme suggests that analogs



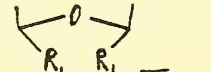
of S-adenosylhomocysteine (AdoHcy), I, might directly inhibit a methylation reaction by binding to a methyltransferase enzyme or indirectly by inhibiting all methylations by binding to AdoHcy hydrolase. Inhibition of the latter enzyme would heighten the intracellular concentration of AdoHcy, which acts as a feedback inhibitor of all methyltransferases. The scheme further suggests that analogs of AdoMet might inhibit either the biosynthesis of AdoMet or one of the many methyltransferases.

Dr. Borchardt's recent studies on analogs of both adenosine derivatives have included the following significant findings:

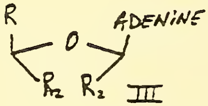
1. Derivative II, referred to as AdoHcy dialdehyde, was synthesized and found to inhibit irreversibly histamine N-methyltransferase (HMT):



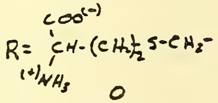
I



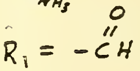
II



III



$R = \underset{(+)NH_3}{\overset{COO^{(-)}}{CH}}-(CH_2)_2-S-CH_2-$

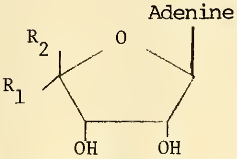


$R_1 = -\overset{\overset{O}{\|}}{C}H$

$R_2 = -CH_2OH$

The enzyme was protected from inactivation by AdoMet, indicating that the dialdehyde is likely interacting with the active site of the enzyme. Derivative II was not found to inhibit other methyltransferases; this result suggests that it may be possible to inhibit methyltransferases specifically. Derivative II was also found to be a potent irreversible inhibitor of bovine liver AdoHcy hydrolase. As was the case with HMT, the natural substrate, AdoHcy, protected the hydrolase from inactivation. In a related study, derivative III, the diol corresponding to II, was found to be a substrate for AdoHcy hydrolase. The enzyme mediates the oxidation of the diol to the monoaldehyde, which subsequently inactivates the enzyme.

2. A pair of analogs of AdoHcy, Sinefungin, IV, and a related metabolite, V, were found to be potent inhibitors of Newcastle disease virion and vaccinia virion mRNA(guanine-7-) methyltransferase and the vaccinia virion mRNA(nucleoside-2'-) methyltransferase. The significance of this finding derives from the fact that the mRNA from eucaryotes--as well as viruses which infect eucaryotes--contains blocked and methylated structures, represented as $m^7GpppN^{(m)}$, where N is often G or A, at the 5' terminus; these structures have been shown to be necessary for efficient binding of mRNA to the ribosome and subsequent translation of the mRNA to proteins. These experiments, conducted in vitro, led Dr. Borchardt and his collaborators to evaluate the potential of these inhibitors to serve as antiviral agents. They found that these AdoHcy analogs inhibited multiplication of vaccinia virus in mouse L-cells at concentrations where no L-cell toxicity was observed. Furthermore, they found that these and other analogs of AdoHcy exhibited inhibitory effects against Rous sarcoma virus transformations of chick embryo fibroblasts.



Compound	R_1	R_2
IV	H	$-CH_2-CH(NH_2)CH_2CH_2CH(NH_2)CO_2H$
V	-	$=CHCH(NH_2)CH_2CH_2CH(NH_2)CO_2H$

These studies illustrate the approaches Dr. Borchardt employs as well as the potential these inhibitors have to serve not only as experimental probes of enzyme structure and function but also as antiviral agents.

"Cytochrome P450-Dependent Oxidation of Arachidonic Acid"

P01 GM 16488-14 (Estabrook, R.), Southwestern Medical School, Dallas

Dr. Ronald Estabrook and his colleagues have had a longstanding interest in obtaining a better understanding of the mechanism and regulation of the enzyme system, cytochrome P450, which is responsible for the oxidative biotransformation of many drugs and other xenobiotics. This enzyme system appears to have multiple functional activities, including oxygenase, peroxidase and oxidase activity. The unsaturated fatty acid of highest content in the phospholipids of rat liver microsomes is arachidonic acid. Recent work in this laboratory has been directed to evaluating the existence and the mechanism of arachidonic acid metabolism by hepatic microsomal cytochrome P450 and with the purified and reconstituted P450 system obtained from porcine liver or kidney (in collaboration with Dr. B. S. Masters). Arachidonic acid is oxidized by the NADPH-dependent hepatic microsomes to a number of oxygen-containing products which are resolvable by HPLC. Several of these products have been purified and characterized. They exhibit an absorbance in the UV region of the spectrum with a maximum at 235 nm, indicative of the presence of a conjugated diene function. Mass spectral analysis of the trimethylsilyl ether derivative of the methyl esters of the hydrogenated and nonhydrogenated metabolites shows that they are the 9-,11-,12-, and 15-monohydroxy derivatives of arachidonic acid, the hydroxyeicosatrienoic acids (HETEs). Their UV absorbance and chromatographic properties suggest that these products possess cis,trans-diene geometry characteristic of HETEs isolated from other mammalian sources. In all, these HETEs plus the 15-keto derivative of arachidonic acid account for about 25 percent of all the organic soluble products of the cytochrome P450 reaction.

In addition, Dr. Estabrook's colleagues have isolated and chemically characterized four novel epoxy derivatives of arachidonic acid produced by the hepatic microsomal system. Using chromatographic and mass spectrometric techniques in conjunction with synthetic methodology to produce the putative molecular structures, these compounds were identified as 14,15-epoxy-5,8,11-eicosatrienoic acid, the major metabolite in this group as well as in the total oxidative metabolism of arachidonic acid by cytochrome P450, 11,12-epoxy-5,8,14-eicosatrienoic acid, and 8,9-epoxy-5,11,14-eicosatrienoic acid. The fourth derivative is still under investigation.

Since none of the epoxy acids contain a conjugated double bond functionality in their structure, it is tempting to speculate that they are formed by an oxygen addition process, probably via an oxenoid type of mechanism. These products, along with the HETEs, are produced by an enzymatic reaction directly coupled to the cyclic function of cytochrome P450. Dr. Estabrook and his group speculate that the diversity of oxidation products of arachidonic acid obtained represents another example of the mechanistic duality of cytochrome P450 in its role as the terminal oxidase of the microsomal electron transport chain. Cytochrome P450 may play a role in the oxidative metabolism of arachidonic acid to physiologically and pharmacologically important hydroxylated unsaturated fatty acids.

"Biosynthetic Intermediates of Porphyrins"
R01 GM 11973-16 (Frydman, B.), University of Buenos Aires

Porphobilinogen deaminase transforms porphobilinogen (PBG) into the metabolic precursors of heme; and porphobilinogen-oxygenase, a mixed function oxidase that catalyzes the oxidation of PBG, diverts it from this metabolic pathway. Evidence suggests that PBG deaminase is involved in the regulation of heme synthesis, since it fluctuates in porphyrias and disappears before the onset of senescence in plants. Further experimental evidence indicates that PBG-oxygenase also exerts regulatory control at this stage of porphyrin biosynthesis since it is induced in the cases when PBG formation exceeds the normal physiological levels. PBG-oxygenase, a non-heme iron-sulfur protein, is a microsomal enzyme in rat liver. It is liberated from the microsomes by treatment with deoxycholate, and has been shown to have sigmoidal-type kinetics. PBG-oxygenase from human blood, however, is entirely a soluble enzyme with kinetics of either the Michaelis or the sigmoidal type, depending on the source of the blood sample. This ambiguity, as well as the meaning of the existence of the human blood enzyme in different ionic forms, has been the subject of investigations by Drs. Benjamin and Rosalia Frydman. Blood from normal rats is entirely devoid of PBG-oxygenase activity unless the rats are kept under conditions of erythropoiesis (e.g. hypoxia).

The enzyme appears in the rat blood at the tenth day of hypoxia. This blood enzyme is soluble, strongly cationic and has Michaelis kinetics. In phenylhydrazine-treated rats, PBG-oxygenase isolated from blood is also a soluble enzyme, but it has sigmoidal kinetics. The best explanation for this discrepancy is that the blood of phenylhydrazine-treated rats contains fewer mature reticulocytes than the blood of rats kept under hypoxia. In phenylhydrazine-treated rats, the enzyme is present in a more "native" state, similar to that of the bone marrow from which the immature cells are released into the blood stream.

PBG-oxygenase can be detected in the bone marrow of normal rats. There is an increase in the enzymatic activity in bone marrow of phenylhydrazine-treated rats as compared to the controls. A study of the properties of the bone marrow enzyme indicates that it is more anionic than the rat blood enzyme. The marrow enzyme has sigmoidal kinetics rather than Michaelis type. The enzymes of bone marrow and blood also differ in their pH optima. The latter has an optimum of pH 7.8-8 while the former has an optimum of pH 7.3. The bone marrow enzyme is microsomal and when the normoblasts are transformed into reticulocytes which are released into the blood stream, the enzyme is very likely liberated from the microsomes, probably as a result of the latter's degradation. Since the blood enzyme has its origin in the bone marrow, the investigators postulate that there must be a change induced by the erythropoietic stress in the bone marrow PBG-oxygenase when the latter is released within the reticulocytes into the blood stream and that this change leads to an enzyme with different properties, which is perhaps in an incipient state of degradation.

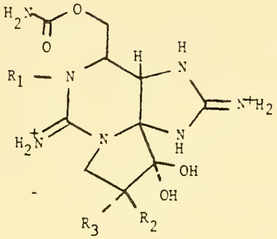
"Deleterious Microalgal Toxins"

R01 GM 28754-08 (Shimizu, Y.), University of Rhode Island

Paralytic shellfish poisoning (PSP) has been a serious problem for a long time in many parts of the world. The sporadic and unpredictable outbreaks usually cause serious health hazards and great losses to the seafood industry. The toxins accumulate in shellfish as a result of ingestion of toxic dinoflagellate. Saxitoxin, first isolated from Alaska butter clams, *Saxidomus giganteus*, and later from California mussels, *Mytilus californianus*, was thought to be the only toxic principle produced by the causative organism *Gonyaulax catenella* (Schantz et al 1966). Recent studies by Dr. Yuzuru Shimizu and his colleagues have shown that the toxicity is caused by a group of closely related compounds and that saxitoxin did not even constitute the major component in many cases. Cultures of *Gonyaulax tamarensis*, the causative organism of PSP in the North Atlantic region, were also studied in their laboratory and found to contain more than seven toxins, including saxitoxin.

The toxicity of saxitoxin is extremely high. By definition there are 5500 mouse units in one milligram of saxitoxin dihydrochloride using the official AOAC assay method. This means that 180 nanograms will kill a mouse under standard conditions. For the other toxins there is no description of their specific toxicities except for two toxins isolated from toxic scallops by Boyer. There is, however, no complete list of specific toxicity of all the toxins determined under the same standardized conditions. Such specific toxicity data are essential in order to develop physicochemical assays of the PSP toxins. Also, interest in saxitoxin and other PSP toxins has been growing rapidly since these molecules proved to be of great value for studying the structure of the sodium channel of the excitable membrane (Narahashi).

Structures of the PSP and their relative toxicities are shown.



	R_1	R_2	R_3	Relative Toxicity
Saxitoxin	H	H	H	100
Neosaxitoxin	OH	H	H	43
Gonyautoxin-I	OH	H	OSO_3^-	72
Gonyautoxin-II	H	H	OSO_3^-	36
Gonyautoxin-III	H	OSO_3^-	H	103
Gonyautoxin-IV	OH	OSO_3^-	H	30

Cultures of *G. tamarensis* have been established in Dr. Shimizu's laboratory, and they were the source of the toxins tested. These cultures are also being used in studies of the biosynthesis of the toxins in collaboration with Dr. Floss of Ohio State University. To date it has been shown that glycine and the *guanido* carbon of arginine are incorporated well into the toxin molecules formed by intact *G. tamarensis* cultures. Further studies are under way to specifically locate the isotopes in the biosynthesized molecules and to deduce the pathways of biosynthesis.

"Pharmacology of Membrane-Associated Receptors"
R01 GM 24337-06 (Taylor, P.), University of California, San Diego

The sequential processes which link formation of a complex between neurotransmitter and its receptor to the resulting physiologic event are being elucidated among others by Dr. Palmer Taylor and co-workers, who have been active in this area of investigation for a number of years. Utilizing a membrane preparation enriched in acetylcholine receptors, purified from the electric organs of the electric ray, *Torpedo California* by gradient sedimentation and affinity partitioning, Dr. Taylor and colleagues have made significant contributions to knowledge of the receptor-neurotransmitter interactions.

Previous studies in this area of investigation have yielded exciting results. Several cholinergic ligands have been shown to have the ability to induce a state transition in the membrane-associated cholinergic receptor where there is a slow increase in affinity for the associating ligand. All of the agonists tested, as well as certain antagonists, notably the metaphilic antagonists i.e., diphenyldecamethonium and dinaphthyldecamethonium, converted the receptor to a higher affinity state. Moreover, the rate of receptor conversion appeared to be independent of the ligand's capacity to activate the receptor. By applying the two-state cyclic scheme used to explain desensitization to these affinity increases, the apparent binding constants of each ligand for the activatable (R), and non-conducting (R') receptor states were determined. Full agonists were found to have a 300-fold higher affinity for the R' state while the classical antagonists (i.e., d-tubocurarine and gallamine) had the same affinity for both receptor states. The metaphilic antagonists (i.e., diphenyldecamethonium) *in situ* showed enhanced antagonism for receptor previously conditioned by agonist. In addition, they also effected a conversion in the receptor state.

Dr. Taylor's current investigation concerns simultaneous measurements of receptor occupation and the change in ion permeability elicited by the ligands. These measurements define the linkage between agonist association and activation of the ion channel.

The decrement in functional capacity of the nicotinic receptor on intact BC3H-1 cells was simultaneously compared with the fractional occupation of the receptor by cobra α-toxin. A parabolic concave inward relationship between the fractional occupation of receptors by α-toxin and the decrement in permeability response obtains when the latter is tested over a range of agonist concentrations. Since α-toxin binding appeared to be equivalent at each site on the receptor, the observed relationship was described by a model where activation of a permeability response required agonist occupation of two toxin-binding sites per functional receptor. Furthermore, the binding of α-toxin and agonist appear to be mutually exclusive, but occupation of either of the two sites by α-toxin is sufficient to block the functional capacity of the receptor. Consistent with this model, when a major fraction of sites is occupied by α-toxin, the concentration dependence for either carbamylcholine-mediated activation or desensitization of the remaining functional receptors is not detectably altered and retains positive cooperativity. In contrast, progressive occupation of the available sites by α-toxin leads to a decrease in apparent affinity and a corresponding loss of positive cooperativity for agonist occupation functions generated upon instantaneous or following equilibrium exposure to the agonist. At high degrees of fractional occupancy by α-toxin, where the dominant species capable of binding agonist would contain a single bound toxin molecule, the Hill coefficient for the equilibrium occupation function for full agonists falls from a value of 1.4 to 0.7. By contrast, the binding isotherms for antagonists which typically exhibit values less than 1.0 are not altered following fractional irreversible occupation by α-toxin. Thus, the two binding sites on the receptor oligomer are interpreted to be not intrinsically equivalent for the binding of agonists and reversible antagonists.

This information will be important in the design of future drugs which act via cholinergic receptors such as muscle relaxants in anesthesiology (curare, pancuronium, succinylcholine), muscle relaxant reversing agents (neostigmine) and in disease states such as myasthenia gravis and atypical pseudocholinesterase.

Pharmacological Sciences

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PHYSIOLOGY AND BIOMEDICAL ENGINEERING PROGRAM

SCOPE

The Physiology and Biomedical Engineering (PBME) Program was established in 1978, by the consolidation of several areas of research previously supported through separate programs of the Institute. Understandably, therefore, its interests are broad in scope, encompassing research on the application of engineering, physical, chemical, biochemical, and physiological sciences to the solution of significant biomedical problems, as well as the development of new instruments and methods to facilitate such research. Studies supported by the program range from laboratory research on cells and tissues to clinical research, and include certain aspects of the behavioral sciences. In addition, the program supports the research training of a number of potential future scientific investigators in areas related to its research interests.

OBJECTIVES

Major objectives of the program are twofold. The first is new knowledge and understanding of biologic function at the organ and organismic levels in order to better enable the physician to prevent, diagnose, and treat a variety of clinical conditions, including those following trauma or burn injury. The second objective is the development of new instruments and methods to advance the biomedical research supported by the Institute.

Support for the accomplishment of the program's research objectives involves the utilization of a variety of mechanisms, including traditional research project grants (R01), program project grants (P01), research center grants (P50), research career development awards (K04), and new investigator research awards (R23). Support for research training is provided through Institutional (T32) and Individual (F32) National Research Service Awards.

The Program is directed by Dr. Leo von Euler, with Dr. Frederick P. Ferguson serving as Program Coordinator. Major organizational components consist of two sections: the Biomedical Engineering and Instrument Development Section, and the Physiological Sciences Section. The professional staff of the former consists of Dr. W. Sue Badman, Chief, and Drs. Marvin Cassman, Americo Rivera, and Bert Shapiro, program administrators. Dr. Matti Al-Aish, formerly a member of this section, transferred to the National Cancer Institute in February 1982. The Physiological Sciences Section, of which Dr. von Euler serves as Acting Chief, is comprised of Drs. Emilie Black, Elizabeth O'Hern, William Taylor and Lee Van Lenten, program administrators

The total budget for the program for Fiscal Year 1982 approximated $37 million, a decrease of some $7.6 million from the Fiscal Year 1981 level. A substantial portion of the decrease ($4.4 million) resulted from the transfer of the program's portfolio of 29 research grants in diagnostic radiology research to the National Cancer Institute's Division of Cancer Treatment. This transfer was a consequence of long-term planning efforts on the part of the two Institutes, as well as numerous discussions with Dr. Donald Fredrickson, former Director of the National Institutes of Health, who made the decision that the activities which NIH supports in the area of diagnostic radiology would be best administered if they were focused in one institute. The National Cancer Institute seemed a logical choice. This decision was consistent with the findings of a special committee of experts that had made an independent study of the matter in the recent past. It was formalized by Dr. Thomas Malone, Acting Director, NIH, in November 1981.

Institute support for the shared instrumentation initiative, begun in Fiscal Year 1979, was continued in Fiscal Year 1982. Awards were made as supplements to the principal investigators' research grants. One such award was made in the PBME Program.

RESEARCH

1. BIOMEDICAL ENGINEERING AND INSTRUMENT DEVELOPMENT SECTION

The goals of this section include the application of physical, chemical, and engineering knowledge to the solution of significant biomedical problems, as well as the development of new instruments, devices, or techniques for basic, physiological, and clinical research. In addition to supporting innovations in instrumentation, this section is concerned with the development of supportive methodologies in the areas of cellular and molecular biology, biochemistry, biophysics, and imaging of biologic structures.

Examples of current research supported by the section include the development of chemical and physical separation techniques, spectroscopic techniques and their extension to the study of ultrafast molecular phenomena, ultrasound and its effects on biological systems, and microscopy involving all types of radiation. Other examples are physiological systems analysis, biomechanics, and physiological instrumentation.

Shared Instrumentation

The NIGMS program for support of shared instrumentation was originally announced in January 1979, as a one-time program. It was reannounced in October 1980, as an ongoing program. The first group of 79 awards, totaling $9 million, was made in Fiscal Year 1979 and Fiscal Year 1980. Most of these awards are nearing termination. A subset of this group of awards is being site visited to develop a set of evaluation criteria.

As a result of the reannouncement, 117 applications were reviewed by the October 1981 Council. Of these, 10 awards totaling $1.3 million were made by the Institute in Fiscal Year 1982 with a number of excellent applications remaining in competition. The third round of applications included 13 received for the October 1982 Council, as well as approximately seven on which NIGMS is the secondary assignee. These applications will compete for Fiscal Year 1983 funds.

2. PHYSIOLOGICAL SCIENCES SECTION

Trauma and Burns

The principal objective of the trauma and burns program is the support of research on the physiologic and pathologic mechanisms of the body's systemic response to injury. The overall goal is to better understand the fundamental biological responses to injury so that more purposeful treatment becomes possible—treatment which capitalizes on the body's homeostatic responses, and which minimizes the untoward effects of the initial injury and the body's over-reactions or inappropriate responses to these injuries.

The trauma and burns research programs supported by the Institute cover a broad spectrum of investigative interests, reflecting needs and promising opportunities. Since the body's response to trauma involves multiple biological systems, the research supported tends to be multidisciplinary, often incorporating the latest ideas and techniques of the clinical disciplines of surgery and medicine, as well as those of the basic disciplines of biochemistry, cell biology, microbiology, immunology, physiology, and biomedical engineering. Besides the complexity of the processes being studied, exploration of the basic responses to severe injury is hampered by the limited number of traumatized patients appropriate for study, the impossibility of "scheduling" studies to be done in the acutely injured individual, and the fact that the clinical condition of the patient often changes rapidly. On the other hand, the use of animals, tissue slices, or cultured cells must balance the more limited applicability of model systems to the human condition against the ability to control and manipulate a greater number of variables in a predictable manner. Despite these problems, impressive improvements in reducing morbidity and mortality from trauma and burns have been made since the program was started in 1966. These advances include complete fluid resuscitation within 24 hours of the injury, parenteral feeding regimens that minimize protein wasting, new skin grafting techniques and skin substitutes that minimize wound infection and scarring, and better anesthetic management and maintenance of adequate respiratory function after injury and operation. Currently the emphasis of most of the research programs being supported falls in one of four general areas: metabolism and nutrition, cellular and organ physiology, immunology and infection, and wound healing. Some examples of ongoing research include the effect of parenteral nutrition on host defense mechanisms, protein turnover, and energy balance; changes in cellular and mitochondrial function induced by shock and trauma; the role of oxygen metabolites in post-traumatic acute respiratory failure; the study of injury-induced immune dysfunctions that cause an increased incidence of anergy, sepsis and death; collagen metabolism in wound repair and keloid formation; and development of an artificial skin.

A variety of grant mechanisms, tailored to the needs of the investigator or project, are used to support trauma and burns research. The regular research project grant (R01) is the most commonly used mechanism with 65 grants currently funded. Research programs are also supported by program project (P01) and research center (P50) grant mechanisms. Currently three program projects and nine centers are funded. These large grants, each involving up to eight interrelated basic research projects, have been placed in the setting of large trauma treatment centers where trauma patients are readily available as research subjects. Two additional mechanisms are designed to encourage recently trained investigators to engage in trauma and burns research. The New Investigator Research Award (R23) seeks to facilitate the physicians's transition from research training to independent scientific investigation, whereas the Research Career Development Award (K04) allows investigators to concentrate on research at the crucial, early state of their careers by providing salary support for a five-year period. Eleven new investigator and two career development awards are currently funded.

Anesthesiology

An important goal of this program is to increase the efficacy and safety of anesthetic agents in man. Toward this end, it emphasizes research on anesthetic metabolism and toxicity, the mode of action of general and local anesthetics, and the development of new anesthetics including neuromuscular blocking agents. In currently active grants, good progress is being made toward an understanding of malignant hyperthermia, a potentially catastrophic syndrome precipitated by certain anesthetics. Productive research is also being conducted on organ protection during anesthesia, with various investigators addressing problems related to the brain, heart, kidney, lung, and liver. The scope of investigations aimed at protecting the anesthetized patient has been further extended to include studies of the effects of anesthetics on viral reproduction and on the possible induction of metastases by anesthesia and surgery. Studies of anesthetic-cell membrane interactions embrace a wide variety of biological systems, including the marine mollusk, *Aplysia* and the squid, *Lologunculus*. Artificial membranes are being used by one investigator to examine the physical aspects of the membrane-anesthetic interaction.

Behavioral Sciences and Adaptation

The objective of this program is to support research on certain fundamental aspects of behavioral sciences, including studies that relate to the clinical research on trauma and burns, and anesthesiology. Among these grants, significant progress has been made in a study involving the specification and classification of *pain and pain patients*. The project will now have an enlarged emphasis which extends the basic research to clinical patients. A research area of considerable interest and activity deals with *circadian rhythms*, where the light-dark entrainment of the rhythms and the properties of a complex pacemaker have received a major thrust. New efforts also have been opened to study the physiological basis of circadian organization, as well as the neural-hormonal control of circadian behaviors. *Brain-behavior studies* are providing for an analysis of the behavioral-neural substrate mediating water balance revealing that peripheral, visceral, afferent fibers are important in water balance where traditionally central mechanisms were given almost exclusive emphasis. Other neurobehavioral systems research addresses problems of brain lesions and salt appetite where it has been found that brain lesion effects on salt intake regulation can be modified significantly by subtle pre-operative learning experiences.

The anatomical, biochemical and biophysical determinants of behavior are being analyzed, using relatively simple animal models at this stage. These studies are being supplemented significantly by genetic approaches.

Three types of award mechanisms are used: regular research projects, program projects, and individual fellowships.

RESEARCH TRAINING

Predoctoral

The training program in Systems and Integrative Biology (SIB) plays an important role in the NIGMS training picture. As part of the PBME Program, it has provided predoctoral training in both physiology and biomedical engineering.

Programs are multidisciplinary but may emphasize one or the other discipline. The Institute has been insistent that biomedical engineering programs provide a strong biological orientation so that trainees may fully enter the world of biomedical research. There has been a similar emphasis on appropriately quantitative training for the more physiologically oriented trainees. The SIB training also has a place in the overall Institute training approach. The SIB training programs concentrate on biomedical problems above the cellular level, in many respects taking up where the Cell and Molecular Biology training grants leave off. There is often a meshing of the neurobiological interests of many SIB preceptors with the interests of preceptors in the Pharmacological Sciences training program. Since several of the SIB programs have a neurobiological emphasis, appropriate for a program stressing integration, regulation and interactions, the trainees may continue in research areas not supported by NIGMS. This is consistent with the NIGMS intent to prepare basic researchers for all biomedical areas, including those relevant to the categorical institutes.

The physiology programs, which have been an important component of SIB grants, have shown significant changes over the past several years. The number of students applying to graduate programs appears to be declining. This decrease is not observed in all graduate physiology departments, however, and in several institutions the quality of the entering student body is as good as or better than in past years, despite a decrease in applications. At the same time, neurobiology appears to attract some of the best graduate students in biomedicine. Students of highest quality are still seeking out all areas of neurobiology: developmental; behavioral; and central processing. When the SIB program was established, some concern was expressed that students in the more physiological graduate programs might not be seeking and obtaining training which was sufficiently quantitative. These concerns are turning out to be largely unfounded. The pervasiveness of computers in modern laboratories has enabled researchers to use more sophisticated data gathering, modeling and simulations than ever before. The good graduate students actually lead the way in computer use and, far from avoiding quantitative approaches, are seeking them out. Modern students are leaders in the electronic revolution. The committees reviewing SIB grant applications in the past fiscal year found several new and renewal proposals of high to outstanding quality. It is apparent that the best SIB programs are comparable in every aspect of quality to the best training programs in the other areas supported by NIGMS.

We noted last year some of the problems which reviewers observed in the bioengineering training programs. It is clear that bioengineers must have large course loads. The reviewers also remarked on the difficulty of the transition from engineering, where one is trained to solve problems, to biomedical sci ence, where one searches for problems. Moreover, the bioengineering students tend to focus on narrow clinical questions. Despite these drawbacks, the engineer can bring an insight into the notably exploding world of technology. This technological understanding and application is beyond the reach of most biomedical scientists. Therefore, it is worth the effort to foster the best programs and make clear to the bioengineering community what can be done to overcome its special problems.

Postdoctoral

Research training at the postdoctoral level is supported through Institutional and Individual National Research Service Awards. Institutional awards (T32s) are provided for training in two areas: Anesthesiology, and Trauma and Burns. Eligibility for stipends in the former area is limited to individuals with an M.D. degree; in the latter it includes those with either the M.D. or the Ph.D. A multidisciplinary approach to training is emphasized in the programs, and it is expected that in most instances at least two years of the postdoctoral experience will be in appropriate basic science departments.

The level of funding for these programs has remained fairly stable over the past three years, and in Fiscal Year 1982 approximated $282,000 for Anesthesiology and $578,000 for Trauma and Burns research. Currently there are five active programs in the former area, supporting 11 trainees. In the Trauma and Burns category there are ten programs with a total of 30 trainees, 80 percent of whom hold the M.D. degree.

Individual National Research Service Awards (F32s) are offered for postdoctoral training not only in Anesthesiology, and Trauma and Burns Research, but also in Clinical Laboratory Sciences, Systems and Integrative Biology, Behavioral Sciences, and Epidemiology. Although modest in size (13 currently active awards), the cadre of fellows supported for training represents a group of individuals with clear potential for developing into first-class independent investigators.

RESEARCH HIGHLILGHTS

"Chromosomal Flow Cytometry: DNA and Centromers"

R01 GM 25076-04 (Van Dilla, M), Lawrence Livermore Laboratory, Berkeley, California

The machine classification or karyotyping of chromosomes in mammalian cells is an important objective in the field of genetics. It has applications to clinical practice such as prenatal and neonatal screening. It also has potential for basic research endeavors where it would make possible isolation of DNA from a given human chromosome for genetic linkage studies and could aid in cloning specific human genes or sequencing specific sets of human DNA.

In 1974, isolated chromosomes were fluorescently stained and measured for DNA content in a flow cytometer for the first time by Dr. Marvin Van Dilla and his group at Lawrence Livermore Laboratory. Classification, in this approach, was based on total chromosome fluorescence. Chromosomes were isolated from a large number of mitotic cells, stained with a DNA-specific fluorescent dye, and passed one-by-one through an intense laser beam adjusted to excite the fluorescent dye. The total fluorescence for each chromosome was measured and accumulated to form a fluorescence histogram depicting the distribution of chromosomal fluorescence among the chromosomes of the population. The fluorescence of each peak specifies a chromosome group and its area specifies the frequency of occurrence of the chromosomes of that group. Depending on the stain used, the 24 types of human chromosomes have been classified into approximately 15 by this type of flow analysis.

Chromosomes that cannot be distinguished on the basis of their total fluorescence can often be distinguished on the basis of their morphology. For example, human chromosomes such as 9, 10, 11, and 12, which cannot be classified on the basis of total fluorescence, have DNA-based centromeric indices (DNA content of the long arm of the chromosome divided by the total chromosomal DNA content) which allow their discrimination. Some types of infrequently occurring aberrant chromsomes can also be classified by their shape. For example, dicentric chromosomes can be distinguished as having two centromeres.

In order to develop a flow cytometric method which would be ultimately capable of distinguishing each of the human chromosome types, Dr. Van Dilla's group adapted a method developed by Dr. Leon Wheeless (GM 23088-04), slit-scan flow cytometry, which had previously been used to estimate cellular shape. The slit-scan approach allows the collection of morphological information about the chromosome, while it is being scanned. In this approach, isolated fluorescently stained chromosomes flow lengthwise through a thin laser beam. The time variation of the resulting fluorescence is measured as each chromosome passes through the laser beam. In developing the slit-scan flow cytometer for chromosomal analysis, a number of problems such as laser beam shaping, depth of focus, precision of chromosome trajectory, chromosome orientation, and signal/noise problems had to be solved. This approach has been combined with improved DNA staining techniques and sophisticated computer programs to allow for automatic, on-line calculation of centromeric indices and debris rejection. The technique has already been used by their collaborators, to map the location of globin genes in the human genome. It currently allows human chromosomes to be classified into approximately 20 groups and may soon allow the discrimination of each of the 24 types of human chromosomes.

"Structure Determination by Triple Quad Mass Spectrometry"
R01 GM 28254-02 (Enke, C), Michigan State University, East Lansing

The development of new analytical techniques having increased range and sensitivity is an almost indispensable precursor to scientific progress. Witness the development of X-ray crystallography as applied to structure determination of polymer fibers, which led to the understanding of DNA structure; or the evolution of the cell sorter, which has revolutionized immunology. In the area of mass spectrometry, several new advances have transformed the field of analytical chemistry. One of these is the development of the triple quadrupole mass spectrometer (TQMS) by Dr. Christie Enke and his colleagues.

In TQMS, the sample molecule is ionized and mass-selected in the normal manner. The selected ion is then fragmented by collisional activation in a quadrupole reaction chamber and analyzed by a third quadrupole and detector. TQMS can potentially identify all the precursor ions for all the ions in the spectrum, and thus provide structural determination of molecules not readily analyzed by other means. Although his system is still being developed, spectral characterization of the ethanol ion fragments allowed the mechanisms for the formation and fragmentation of these ions to be readily derived.

Although the power of this technique is quite clear, its value will only become apparent as the relationship between compound structure and patterns in the mass spectra is worked out.

"Surgical Implants for Sustained Macromolecular Release"
R01 GM 26698-03 (Langer, R.), Massachusetts Institute of Technology

Many drugs work best when they are administered into the body at a constant rate which maintains their concentration within therapeutic range. Unfortunately this is most difficult to accomplish without causing considerable inconvenience to the patient. In recent years oral forms of medication have been developed that release their drug content over an extended period of time. These, however, are applicable only for drugs which are effective when taken orally. Implantable pumps have been devised to provide drug infusion into the blood stream at constant rates. They, however, have some major disadvantages, not the least of which is the difficulty of keeping them charged with the drug being infused.

Dr. Robert Langer has been interested in developing an implantable drug delivery system that can be controlled. He found that a co-polymer of ethylene-vinyl acetate was excellent for embedding proteins. It did not elicit any biological responses, and it allowed the protein to diffuse into its surroundings. Furthermore, by studying the release of proteins from pellets of different shapes, Dr. Langer and his colleagues found that a specially designed hemispheric pellet would release its drug contents at a constant rate from start to finish. The major breakthrough came, however, when Dr. Langer found that he could change the rate of release of a drug from a special polymer slab by subjecting it to an oscillating magnetic field. The special polymer protein slab was constructed to contain stainless steel spheres suspended within the polymer protein matrix and not be in contact with biological tissue. When subjected to an oscillating magnetic field, the spheres caused the rate of protein release from the polymer matrix to increase. These investigators hope to prepare a polymer with a very low background diffusion rate for a drug which upon modulation in a magnetic field would release the drug at a significantly higher rate. In effect, the investigators feel they have developed an implantable polymer drug capsule which can be turned on or off.

"A Study of Wound Healing and Wound Infection"
P50 GM 27345-02 (Hunt, T.), University of California, San Francisco

Trauma research often has stimulated areas of development which impact on a wide variety of other diseases. A case in point is the study by Dr. Thomas Hunt, director of an NIGMS trauma research center, of a naturally occurring factor that stimulates angiogenesis. In the past, several commercial laboratories have been interested in a chemical substance which causes blood vessels to grow toward tumors. Using fluid drawn from steel wire mesh cylinders implanted in the flanks of New Zealand white rabbits, Dr. Hunt and collaborators have been able to isolate and purify a relatively small molecular weight protein which has such potent blood vessel growth-stimulating properties that it has been possible to make blood vessels grow in fiberglass filter paper.

The clinical needs for such a substance are numerous. For instance, it could stimulate healing in patients with a leukopenia who need to take steroids or who have had operations on areas of the body where X-ray therapy had been previously administered. There is a need to stimulate blood vessel growth into the artificial skin grafted on burn patients or into marginal wounds in trauma patients in order to promote rapid healing and resistance to infection.

Also, new blood vessel formation should stimulate the fixation of artificial devices.

Dr. Hunt believes the chemical can be isolated in large quantities. He feels that as soon as an antibody is prepared, one should be able to determine if it is the same substance that permits tumors to attract blood vessels. Since many tumors require an ample blood supply for nutrition, it could be an important anti-tumor agent.

"Organ and Cellular Response to Trauma and Burns"'

P50 GM 24990-04 (Carrico, C.J.), University of Washington, Seattle

At the 1978 NIGMS Consensus Development Conference on Supportive Therapy in Burn Care, the participants concurred that the use of steroids was never indicated in the treatment of smoke inhalation. At the 1980 follow-up conference sponsored by NIGMS, some support was engendered for a controlled prospective field study of the use of a single injection of steroids to provide protection against injury, but the opinion was unanimous that prolonged use would be contraindicated.

Dr. C. James Carrico and his team of investigators at the University of Washington Trauma Research Center have been studying the effects of steroids on animals. They have showed that there is a potential benefit from administration of steroids for isolated smoke inhalation without concomitant thermal cutaneous injury. Until recently the effects have not been examined in the clinical setting. However, fortuitous circumstances surrounding recent hotel fires in Nevada resulted in a data base of a large number of individuals with similar exposure to smoke. With the cooperation of several hospitals in the vicinity of the fires, the team of investigators was able to identify two groups of patients with sufficient data to allow rigorous statistical comparison. One group had been treated with single injections of steroids, the other had received no steroids. The two groups were matched for age, sex, presenting symptoms, medical history, laboratory data, and final outcome. There were no detectable differences between treated and nontreated groups. Arterial blood gases were comparable and this relationship did not change significantly for the remainder of the study period. Likewise, pulmonary morbidity was compared and there were no hospital mortalities in either group. The conclusion was that single injections of steroids have little effect on pulmonary-related morbidity and mortality following smoke inhalation.

"Role of Endorphins in Anesthetic-Induced Analgesia"

R01 GM 26407-02 (Finck, A. D.), Columbia University, New York

The role of endogenous opioids and of the opiate receptors in analgesia has been of great interest since the discovery of the receptors in 1973, and the demonstration of enkephalins and endorphins two years later. Identified on the basis of their ability to react with receptors that mediate responses to morphine and other opiates, these neuropeptides, (β endorphin and other endogenous opioids) can be antagonized by drugs such as naloxone which prevent or reverse the agonist effect. It has been demonstrated that analgesia produced by activation of a brain electrode is accompanied by increased levels of endorphins in the cerebrospinal fluid. Naloxone partially antagonizes this analgesia as it does the endorphin-produced analgesia in rats subjected to the stress of inescapable foot shock. Because naloxone has been found to antagonize analgesia produced by acupuncture in mice and in humans, it has been surmised that acupuncture-induced analgesia may be mediated by endorphins.

Studies by Dr. Donald Finck on the relationship of anesthesia to endogenous opioids have utilized analgesia as a semi-quantifiable endpoint for the complex, qualitative state of anesthesia. He found that animals anesthetized with halothane, enflurane, or cyclopropane do respond to a painful stimulus after intravenous administration of naloxone. He also has demonstrated that animals made tolerant to nitrous oxide (N_20) display no crosstolerance to morphine. Conversely, animals made tolerant to morphine did display crosstolerance to N_20. These results are consistent with the view that N_20 causes a release or increased utilization of endorphins while morphine acts at the opiate receptor level (post-synaptically) and would still be effective when given to N_20 tolerant animals. Rats made tolerant to morphine, by whatever mechanism, would not be expected to respond to N_20 if an endorphin-related mechanism is involved, as was demonstrated.

Extension of these results was obtained with ketamine, a non-gaseous anesthetic, which appears to have a similar action. In pharmacologically relevant concentrations, ketamine was found to bind to opiate receptors in in vitro test systems and to displace the radioactively labeled narcotic, ^{3}H-etorphine, from opiate receptors in vivo. These results suggest that a significant mechanism of ketamine-induced analgesia is mediated by opiate receptors.

"Injury-induced Growth and Myelin-derived Growth Factors"
R01 GM 31080-03 (Mescher A.), Indiana University, Bloomington, Indiana

The principal investigator is seeking to evaluate the role of factors released by nerves in the cellular events of tissue repair, and to ascertain whether mitogens derived from myelin are involved in promoting cell proliferation following tissue injury. Dr. Anthony Mescher's study has involved the manner in which peripheral nerves promote tissue repair in crush-injured forelimbs of larval Ambystoma salamanders. While it is well known that amputated limbs of adult salamanders require a neurotrophic influence in order to regenerate the missing parts, larval limbs require nerves even to effect the comparatively minor process of tissue repair. If such limbs have their nerves cut at the brachial plexus and then are injured internally distal to the elbow, they not only fail to repair the crush injury, but undergo a process of cell death and tissue resorption which results in regression of the entire limb until such time that nerves regenerate to the affected tissues.

Dr. Mescher undertook a histological and DNA autoradiographic analysis of this process in order to ascertain the dependence of specific cellular events (dedifferentiation, DNA replication, mitosis, redifferentiation) upon the presence of intact nerves. The extent of tissue dedifferentiation and the percentages of mesodermal cells showing DNA synthesis and mitosis were determined in sets of injured forelimbs every other day for two weeks following bilateral crushing and unilateral denervation. Briefly, both forelimbs showed comparable initial levels of dedifferentiation and DNA labeling, but while the innervated limbs showed peak DNA labeling and mitotic activity on days four to six and a return to baseline levels (indicating limb repair) by day ten, the denervated limbs showed continued high levels of DNA replication with barely detectable levels of mitotic activity.

The interpretation of these results is similar to that discussed in Dr. Mescher's earlier studies: that the failure of denervated urodele limbs to regenerate or repair injuries is *not due* to a failure of the cells to renew or accelerate cell cycling in response to the injury, but *is due* instead to a failure of the cells to complete the cell cycle and divide at normal levels. Presumably, normal mitotic activity in the cells depends on axoplasmic factors released from nerve endings.

CONTRACTS

The support of contracts and interagency agreements constitutes a relatively minor activity of the Physiology and Biomedical Engineering Program.

1. "Reconstruction and Testing of a Superconducting Electron Microscope" (Contract N01 GM 2116), Duke University, ($336,327, Fiscal Year 1982 Funds)

A three phase contract was awarded to Duke University on June 30, 1980, with Dr. J. David Robertson as Principal Investigator and Robert Worsham, the original builder of the superconducting electron microscope, as Project Engineer.

Phase I, which involved reassembly and restoration of the microscope, was successfully completed by March 14, 1982. Phase II, evaluation of the microscope, began March 15, 1982. Drs. Michael Lamvik and David Kopf were appointed to replace Robert Worsham and aid in completion of the contract. Phase II requires the investigators to demonstrate in a systematic way: 1) the improvement in electron exposure that can be tolerated by glucose embedded catalase, or a similar specimen, at liquid helium temperature compared with that obtained at liquid nitrogen and room temperature; 2) that high resolution electron diffraction patterns can be obtained from crystals of biological interest using the superconducting microscope; and 3) whether the instrumental contrast transfer function extends to better than 3 Å. With these results a complete evaluation of the practical potential of the superconducting microscope for contributing new information of high resolution biological structure will be obtained as well as an evaluation of its performance as a potential "user-oriented" instrument. Substantial progress is being made on item 1 above.

Since, if Phase II is wholly successful, the future use of the microscope is expected to be supported through a regular research or research resource grant, an additional phase will provide support for one year, keeping the microscope intact and under vacuum, to allow for an orderly transition to such support.

2. "Refurbishment and Renovation of the Infrared Heterodyne Ratiometer" (Contract N01 GM 52124), Andros Incorporated, ($35,000 Fiscal Year 1980; no Fiscal Year 1982 Funds)

The generally accepted method for assaying the stable isotope of carbon (atomic weight of 13 instead of 12) is by high resolution mass spectroscopy, requiring instrumentation costing in excess of $250,000. NIGMS funds were used to construct a $^{13}CO_2/^{12}CO_2$ ratio analyzer using innovative ideas from NASA-Ames and the Andros Corporation in the hope of developing an alternative analytical instrument and procedure of adequate sensitivity and accuracy, but less costly to manufacture and maintain than the mass spectrometer. This ratiometer is being prepared for use in the laboratory of Dr. Charles Irving, Baylor College of Medicine, who responded successfully to a request for applications issued by the program. This laboratory will use this ratiometer to gather important human data on the production of $^{13}CO_2$ after ingestion of ^{13}C labeled compounds and will validate the new analytical procedure.

Physiology and Biomedical Engineering Program

Program Organization and Professional Staff

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Minority Access to Research Careers

Scope

The Minority Access to Research Careers (MARC) Program was formally established in 1975, and is a unique research training support program administered by the National Institute of General Medical Sciences in collaboration with other institutes of the National Institutes of Health. The activities of the MARC Program for Fiscal Year 1982 are reviewed below.

Objectives

The primary goals of the MARC Program are to increase the number and capabilities of minority scientists engaged in biomedical research and to strengthen science curricula and research opportunities at minority institutions in order to prepare students for careers in biomedical research.

Mechanisms

To achieve these goals, the MARC Program uses an array of research training grants and fellowships. These are: the MARC Faculty Fellowship, the MARC Visiting Scientist Award, the MARC Honors Undergraduate Research Training Program, and the MARC Predoctoral Fellowship.

Program Components

The MARC Faculty Fellowship Program -- the first of the four MARC support mechanisms developed provides opportunities for advanced research training for selected faculty members of four-year colleges, universities and health professional schools in which student enrollments are drawn substantially from ethnic minority groups. These institutions may nominate faculty members for MARC Fellowships for a period of up to three years of advanced study and research training, either as candidates for the Ph.D. degree or as investigators obtaining postdoctoral research training in the biomedical sciences. Faculty Fellows are expected to return to the nominating institutions at the completion of their training.

MARC Faculty Fellows are selected on a competitive basis. Evaluation is based upon the applicant's qualifications and potential for research and teaching, as evidenced by academic records, reference reports and publications, as well as by a research training proposal, the proposed training situation (i.e., the sponsor's and the institution's training facilities and staff), and other relevant information.

The second support mechanism, the MARC Visiting Scientist Award, provides financial support for outstanding scientist-teachers to serve as visiting scientists at four-year colleges, universities, and health professional schools where student enrollments are drawn substantially from minority groups. The primary intent of this award is to help strengthen research and teaching programs in the biomedical sciences for the benefit of students and faculty at these institutions by allowing them to draw upon the special talents of expert scientists from other institutions. Reciprocal benefits should accrue to the

Visiting Scientist through the added experience gained by his or her involvement in innovative science education and research development programs.

The proposal must include arrangements for the visiting scientist to reside in the campus community and to participate fully in programs of teaching, development of research, and/or counseling as outlined by the institution. Evidence of negotiations and of some tentative agreement between the applicant institution and the scientist-teacher nominated to serve as the MARC Visiting Scientist should be provided in the application. The individuals nominated should be recognized biomedical scientist scholars and leaders. Proposals may request support for periods ranging from one academic quarter to a maximum of one year. Stipends are determined on an individual basis, according to the nominee's current salary or other possible source of stipend support for the proposed period in residence.

The MARC Honors Undergraduate Research Training Grant Program, the third mechanism of support was initiated at the suggestion of Congress and Institute consultants and staff, as a means of emphasizing the value and importance of providing biomedical research training at the undergraduate level in minority institutions. The objectives of the program are: to increase the number of well-prepared students who can compete successfully for entry into graduate programs leading to the Ph.D. degree in the biomedical sciences, to help develop a strong undergraduate science curriculum, and to stimulate an interest in undergraduate research as preparation for graduate study in the biomedical sciences.

Training support is offered to carefully selected undergraduate honors students at four-year colleges, universities, and health professional schools in which student enrollments are drawn substantially from ethnic minority groups (such as American Indians, Blacks, Hispanics and Pacific Islanders). Each institutional grant is awarded for a maximum period of five years. These honors programs for third and fourth year students should be designed to improve significantly the research training capabilities of the minority institutions. Applications for support should provide information regarding proposed mechanisms to augment and improve the science curricula, strengthen the faculty, and improve laboratory facilities. Funds are available for research equipment and supplies essential to the program, and for stipends, tuitions, fees, and limited travel costs for the trainees. Arrangements for special training at universities and laboratories other than the grantee institution are encouraged and should be described in the application.

The most recently established component of the program is the MARC Predoctoral Fellowship Program, which provides support for research training leading to the Ph.D. degree in the biomedical sciences for individual selected students who are graduates of the MARC Honors Undergraduate Research Training Program. It is expected that such training will be conducted in graduate-degree programs of the highest quality. Support is not available to individuals enrolled in medical or other professional schools, unless they are enrolled in a combined degree (M.D.-Ph.D.) program. Awards are conditional upon acceptance into a specified doctoral (Ph.D.) degree program in biomedical research.

MARC Predoctoral Fellows are selected on a highly competitive basis. A maximum of five years of support may be recommended, based on the merit of the application and evidence of satisfactory progress in the doctoral program in which a successful applicant is enrolled. The award provides an annual stipend to the student, and funds to help defray the expense of tuition, fees and supplies.

Organization and Management

The MARC Program is administered by its Director, Mr. Elward Bynum and one professional staff member, Mrs. Dolores L. Lowery, as program administrator. In addition, the senior consultant to the Institute, Dr. Geraldine P. Woods, provides ongoing assessment and evaluation of the Program.

Highlights

Since the inception of the MARC Faculty Fellowship Program in 1972, 167 individuals have received support under the program. In this ten-year period, approximately 382 applications for MARC Fellowship support were received. Of the 89 predoctoral and 78 postdoctoral award recipients, 121 have completed their training period. Of the 89 predoctoral fellows, 42 (approximately 47 percent) have now received the terminal degree, and 108 (89 percent) of those who have completed the program (121) have returned either to the original home institution or to another minority institution. Of the 78 postdoctoral fellows, 64 have completed training and 58 (91 percent) have returned to minority institutions.

The research training sites have included 70 universities, research laboratories, and federal institutions in 32 states and 5 foreign countries. The home institutions are broadly representative, including 68 universities in 23 states and the Commonwealth of Puerto Rico.

There has been little change in the level of support for the Visiting Scientist Award Program. Only 7 awards have been provided, based on 14 applications reviewed. The requirement for considerable advance planning by the host institution and the prospective visiting scientist may have tempered a fuller usage of this award. However, concerted efforts to advertise and encourage applications for the Visiting Scientist Award are being made by program staff. The MARC Review Committee and the National Advisory General Medical Sciences Council strongly support continuation of this award mechanism.

To date, 195 applications for the Honors Undergraduate Research Training Program have been received, of which 104 have been approved, and 44 have been awarded to minority institutions around the country. In 1981, there were 109 graduates. In 1982, the program had 154 graduates, significant numbers of whom have applied to graduate schools.

Last year, the newest component of the MARC Program, the MARC Predoctoral Fellowship, was initiated. It had been noted by program staff that a number of students graduating from the Honors Undergraduate Program were reluctant to continue their education outside of a minority college setting, and that they were not as aggressive as other students in seeking out training opportunities.

In 1981, National Institute of General Medical Sciences invited applications for individual National Research Service Award MARC Predoctoral Fellowships to initiate this component of the MARC Program. Sixteen applications were received, of which 12 were approved and 4 funded. This year 29 applications were received, of which 19 were approved and 14 funded.

EVALUATION

Assessment of the improvements and accomplishments of the various components of the MARC Program is a continuing effort of program staff. The scientific community, the MARC Review Committee, the National Advisory General Medical Sciences Council and special Institute consultants cooperate in this effort through continued interaction with program and other NIGMS staff.

Significant accomplishments of the program are the following:

The "honors" concept of the MARC Program has encouraged institutions that are recipients of awards to establish a different and more rigorous program for students in the basic sciences. Many have incorporated an honors thesis requirement for graduating MARC students.

The provision of funds for seminars and lectures, release time for faculty members, up-to-date equipment and supplies, as well as the addition of full-time and part-time faculty in disciplines not formerly represented, have all contributed to academic enrichment. In addition, specialized courses have been set up to prepare students to take standardized tests -- an area where minority students have traditionally been weak.

The scientific activities of grantee departments have been strengthened, and as a result, it has become more common for MARC faculty members and trainees to be invited to present papers at local, regional, and national scientific meetings.

The MARC Program, with its emphasis on research, has produced a new awareness of the possibilities available in biomedical research among students historically motivated towards careers in medicine, dentistry, and education.

The fact that MARC students are performing well in graduate schools, has provided an increased number of "role models" for minority students, and resulted in a 50 percent increase in the number of science majors at minority institutions having MARC awards.

Supplementary Activities

The first MARC Undergraduate Scholars Conference was held on September 30 through October 2, 1981, at the National 4-H Center in Chevy Chase, Maryland. Ninety MARC-supported college seniors attended the conference. Many had prepared poster presentations of their research and spent much of the three-days meeting with graduate school faculty and scientist representatives of the National Institutes of Health (NIH) to discuss opportunities in graduate education. They received information on the graduate school application process, availability of financial aid, and expectations regarding the Graduate Record

Examination. As part of the conference, participants attended scientific seminars and toured the NIH research laboratories. They also heard welcoming remarks by Dr. Thomas E. Malone, Acting NIH Director, and Mr. Elward Bynum, director of the MARC Program.

Dr. DeWitt Stetten, NIH Senior Scientific Advisor, addressed the scholars at lunch on the second day; and Representative Louis Stokes (D-Ohio) spoke at a banquet that evening. During the banquet, a plaque was presented to Dr. Geraldine Woods, special consultant to the National Institute of General Medical Sciences, by Dr. Ruth Kirchstein, Director, in recognition of the many contributions Dr. Woods has made to the MARC Program. Dr. Stetten, who was NIGMS Director when the MARC program was developed in the early seventies, spoke of promising fields for future research and of the relationship between "research and technology -- between ideas and craft." Congressman Stokes pledged his continuing interest in and support of the MARC Program.

On the final day of the Scholars Conference, a concurrent MARC Program Directors' Meeting was held to provide a forum for discussion of the summer research activities either in progress or planned by several research institutions, specifically for MARC students.

Discussions also centered on the submission of renewal applications, predoctoral applications and grants management issues affecting each of the 38 MARC Program Directors present. The Visiting Professors Program, administered by the Federation of American Societies for Experimental Biology and funded by the MARC Program, was presented; and participants were urged to utilize more fully this valuable resource.

Minority Access to Research Careers Program

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Dolores Lowery	Education Specialist

Support Staff

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